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(71) Applicant: Toyo Boseki Kabushiki Kaisha
Osaka-shi Osaka 530 (JP)

(72) Inventors:
• Kitabayashi, Masao,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
• Arakawa, Taku,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
• Inoue, Hiroaki,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)

• Kawakami, Bunsei,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
• Kawamura, Yoshihisa,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
• Imanaka, Takayuki
Suita-shi, Osaka-fu (JP)
• Takagi, Masahiro
Suita-shi, Osaka-fu (JP)
• Morikawa, Masaaki
Minoo-shi, Osaka-fu (JP)

(74) Representative: VOSSIUS & PARTNER
Postfach 86 07 67
81634 München (DE)

(54) A method of amplifying nucleic acid and a reagent therefor

(57) An object of this invention is to provide an enzyme which amplifies nucleic acid with a high fidelity within a short reaction time and also to provide a method of amplification.

This invention relates to a thermostable DNA polymerase having at least 30 bases/second of DNA extension rate and a 3'-5' exonuclease activity derived from an hyperthermophilic archaeon strain KOD1, to a method of amplifying and also of detecting the nucleic acid using said enzyme and to a reagent kit used for those methods.

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Description

The present invention relates to a method of amplifying nucleic acid wherein DNA or RNA is amplified within a short reaction time and with a high fidelity, to a method of identifying nucleic acid utilizing said amplifying method and to a DNA polymerase and a reagent kit used for those methods.

Many studies have been made already for DNA polymerase of mesophilic microorganisms such as *Escherichia coli* and for DNA polymerase derived from phages infectable by the mesophilic microorganisms. In addition, many studies have been also made already for heat stable DNA polymerases which are useful in a recombinant DNA technique by means of nucleic acid amplification such as a polymerase chain reaction (PCR). Examples of the heat-stable polymerases which are used for the PCR are DNA polymerase (Tth polymerase) mostly derived from *Thermus thermophilus* and DNA polymerase (Taq polymerase) derived from *Thermus aquaticus*. Other known examples are DNA polymerase (Pfu polymerase) derived from *Pyrococcus furiosus* and DNA polymerase (Vent polymerase) derived from *Thermococcus litoralis*.

However, with the Taq polymerase, fidelity and thermostability upon the synthesis of DNA are not sufficient. Although the Pfu polymerase exhibiting excellent fidelity and thermostability has been developed, said Pfu polymerase has some problems that its DNA extension rate is slow and a processivity is low whereby it has been used only for a specific PCR.

Recently, a PCR whereby 20 kb or more DNA is amplified (hereinafter, referred to as a long-PCR) has been developed. In said long-PCR, both Taq polymerase and Pfu polymerase are mixed whereby properties of both enzymes are utilized. However, when two enzymes having different properties are used in the same reaction system, some discrepancies might occur in their appropriate reaction conditions whereby there is a question whether the high extension rate and fidelity which are the advantages of each of those enzymes can be still maintained. Moreover, because of the difference in the thermostabilities and in the composition of the stock solutions of both enzymes, there is a question as to the stability when they are stored in the same container.

In view of the above, there has been a keen demand for novel thermostable polymerase which exhibits both of those advantages.

The present inventors have succeeded in preparing a thermostable DNA polymerase from a hyperthermophilic archaeon strain KOD1, and, when its properties are investigated, it has been found that said DNA polymerase exhibits the advantages of the above-mentioned two enzymes, i.e. high extension rate and high fidelity, whereby the present invention has been achieved.

Thus, the present invention relates to a method for amplifying a target nucleic acid comprises reacting the target nucleic acid with four kinds of dNTP and primer complementary to said target nucleic acid in a buffer solution which contains a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity such that the above mentioned primer is annealed to the target nucleic acid and an extension product is synthesized from the primer.

The present invention further relates to a method for amplifying a target nucleic acid in a sample wherein each target nucleic acid consists of two separate complementary strands which comprises the following steps A to D, characterized in that a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity is used as a thermostable DNA polymerase;

A: modifying the target nucleic acid, if necessary, to produce single-stranded nucleic acids;

B: reacting the single-stranded nucleic acids with four kinds of dNTP and primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, in a buffer solution which contains a thermostable DNA polymerase such that the above mentioned primers are annealed to the single-stranded nucleic acids and extension products are synthesized from the primers,

C: separating the primer extension products from the templates on which they are synthesized to produce single-stranded nucleic acids; and

D: repeatedly conducting the above mentioned steps B and C.

The present invention further relates to a method for detecting a target nucleic acid in a sample wherein each target nucleic acid consists of two separate complementary strands which comprises the following steps A to E, characterized in that a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity is used as a thermostable DNA polymerase;

A: modifying the target nucleic acid, if necessary, to produce single-stranded nucleic acids;

B: reacting the single-stranded nucleic acids with four kinds of dNTP and primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, in a buffer solution which contains a thermostable DNA polymerase such that the above mentioned primers are annealed to the single-stranded nucleic acids and extension products are synthesized from the primers,

C: separating the primer extension products from the templates on which they are synthesized to produce single-stranded nucleic acids;

D: repeatedly conducting the above mentioned steps B and C, and

E: detecting an amplified nucleic acid.

5 The present invention further relates to a reagent kit for amplifying target nucleic acid which comprises primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, four kinds of dNTP, divalent cation, thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity and buffer solution.

10 The present invention further relates to a reagent kit for detecting target nucleic acid which comprises primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, four kinds of dNTP, divalent cation, thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity, amplifying buffer solution, a probe capable of hybridizing with amplified nucleic acid and a detection buffer solution.

15 The present invention relates to a thermos table DNA polymerase which is obtainable from a strain KOD1 which belongs to a hyperthermophilic archaeon strain.

The present invention relates to an isolated DNA comprising a nucleotide sequence that encodes the thermostable DNA polymerase derived from a KOD1 strain which belongs to hyperthermophilic archaeon.

20 Preferably, said DNA comprises the nucleotide sequence of SEQ ID No. 5 or a nucleotide sequence encoding the thermostable polymerase which has the amino acid sequence of SEQ ID No. 1.

The present invention also relates to a DNA encoding a polypeptide with the above mentioned biological activities comprising a nucleotide sequence

(a) which differs from the above DNA in codon sequence due to the degeneracy of the genetic code;

25 (b) which hybridizes with the above DNA or the DNA of section (a); or

(c) represents a fragment, allelic or other variation of the above DNA.

30 In this context, the term "hybridization" refers to conventional hybridization conditions (see for example Nucleic acid hybridization, A practical approach, Hames and Higgins, eds., IRL Press, Oxford Washington DC, 1985). Most preferably, the term "hybridization" refers to stringent hybridization conditions.

The present invention also relates to a thermostable DNA polymerase with the above mentioned biological activities and being encoded by one of said DNA's and, in addition, to a kit comprising said thermostable DNA polymerase.

35 The present invention further relates to a recombinant DNA expression vector that comprises the DNA sequence inserted into a vector, wherein the DNA sequence encodes the thermostable DNA polymerase derived from a KOD1 strain which belongs to hyperthermophilic archaeon.

The present invention further relates to a transformed recombinant host cell using a recombinant DNA expression vector that comprises the DNA sequence inserted into a vector, wherein the DNA sequence encodes the thermos table DNA polymerase derived from a KOD1 strain which belongs to hyperthermophilic archaeon.

40 The present invention relates to a method for producing a DNA polymerase obtainable from a KOD1 strain which belongs to hyperthermophilic archaeon, comprises culturing recombinant host cells which is transformed by a recombinant DNA expression vector that comprises the DNA sequence inserted into a vector, wherein the DNA sequence encodes the thermostable DNA polymerase derived from a KOD1 strain which belongs to hyperthermophilic archaeon, and recovering the produced thermostable DNA polymerase.

45 The present invention further relates to a method for purifying the DNA polymerase obtainable from a KOD1 strain which belongs to hyperthermophilic archaeon, comprises culturing the recombinant host cells which is transformed by a recombinant DNA expression vector that comprises the DNA sequence inserted into a vector, wherein the DNA sequence encodes the thermos table DNA polymerase derived from a KOD1 strain which belongs to hyperthermophilic archaeon, and further (a) recovering the cultured recombinant host cells, disintegrating them and preparing the cell extract, and (b) removing the impurified proteins derived from recombinant host cells.

50 The nucleic acid which is to be amplified by the present invention is DNA or RNA. There is no restriction at all for the sample in which such a nucleic acid is contained.

55 The thermostable enzyme which is used in the present invention is a thermostable DNA polymerase having at least 30 bases/second of DNA extension rate and having a 3'-5' exonuclease activity. Its specific example is a DNA polymerase derived from a hyperthermophilic archaeon strain KOD1 (called a KOD polymerase) and said enzyme may be either a thermostable enzyme purified from nature or an enzyme manufactured by a gene recombination technique.

The DNA extension rate in the present invention is calculated from the relationship between the reaction time and the size of the synthesized DNA in the reaction of various kinds of DNA polymerases such as KOD, Pfu, Deep Vent, Taq, etc. (5U) in each buffer using a substrate prepared by annealing a single-stranded DNA (1.6 µg) of M13 with a primer (16 pmoles) complementary thereto. It is essential in the present invention that the DNA extension rate is at least

30 bases/second.

The DNA extension rates for each of the polymerases are 105-130 bases/second for KOD polymerase, 24.8 bases/second for Pfu polymerase, 23.3 bases/second for Deep Vent polymerase and 61.0 bases/second for Taq polymerase.

On the other hand, it is essential in the present invention that the thermostable DNA polymerase has a 3'-5' exonuclease activity.

In the present invention, the 3'-5' exonuclease activity is determined by checking the rate of release of ^3H under the optimum condition for each polymerase using a substrate wherein the 3'-end of the lambda-DNA digested with HindIII labeled with [^3H]TTP.

In the 3'-5' exonuclease activity of each polymerase, free- ^3H is found to be only 10-20% in the case of Taq polymerase and Tth polymerase after an incubation period of three hours, in KOD polymerase and Pfu polymerase, it is 50-70%.

It has been confirmed that the KOD polymerase used in the Present invention has a 3'-5' exonuclease activity and that, in the gene which codes for KOD polymerase, there is a DNA conserved sequence showing a 3'-5' exonuclease activity the same as in the case of Pfu polymerase.

In the present invention, the fact whether there is a 3'-5' exonuclease activity is checked in such a manner that KOD polymerase is allowed to stand, using a DNA fragment into which the DNA of [^3H]TTP-labelled-lambda-DNA digested with HindIII is incorporated as a substrate, at the reaction temperature of 75°C in a buffer (20mM Tris-HCl of pH 6.5, 10mM KCl, 6mM $(\text{NH}_4)_2\text{SO}_4$, 2mM MgCl_2 , 0.1% Triton X-100 and 10 $\mu\text{g/ml}$ BSA) and the ratio of the free-[^3H]TTP is determined.

At the same time, Taq polymerase and Tth polymerase having no 3'-5' exonuclease activity and Pfu polymerase having a 3'-5' exonuclease activity were checked using a buffer for each of them by the same manner as in the control experiments. The titer of each of the used polymerases was made 2.5 units.

The substrate DNA was prepared in such a manner that, first, 0.2 mM of dATP, dGTP, dCTP and [^3H]TTP were added to 10 μg of lambda-DNA digested with HindIII, the 3'-end was elongated by Klenow polymerase, then DNA fragments were recovered by extracting with phenol and precipitating with ethanol and free mononucleotides were removed by a Spin column (manufactured by Clontech).

In the case of KOD polymerase and Pfu polymerase, 50-70% of free [^3H]TTP were detected after an incubation period of three hours, in the case of Taq polymerase and Tth polymerase, only 10-20% of free [^3H]TTP was noted.

It is preferred that said thermostable DNA polymerase contains an amino acid sequence given in SEQ ID No.1.

It is also preferred that said thermostable DNA polymerase is an enzyme having the following physical and chemical properties.

Action: It has a DNA synthetic activity and a 3'-5' exonuclease activity.

DNA extension rate: at least 30 bases/second

Optimum pH: 6.5-7.5 (at 75°C)

Optimum temperature: 75°C

Molecular weight: about 88-90 Kda

Amino acid sequence: as mentioned in SEQ ID No.1

An example of the methods for manufacturing DNA polymerase derived from a hyperthermophilic archaeon strain KOD1 is that thermostable DNA polymerase gene was cloned from strain KOD1 which was isolated from a solfatara at a wharf on Kodakara Island, Kagoshima so that a recombinant expression vector was constructed, then a transformant prepared by transformation by said recombinant vector was cultured and the thermostable DNA polymerase was collected from the culture followed by purifying.

In the present invention, the DNA polymerase derived from the above-mentioned hyperthermophilic archaeon strain KOD1 has a DNA synthesizing activity and a 3'-5' exonuclease activity and has a DNA extension rate of at least 30 bases/second. This property is used for conducting an amplification of nucleic acid

The amplifying method of the present invention includes the following steps A to D.

A: modifying the target nucleic acid, if necessary, to produce single-stranded nucleic acids;

B: reacting the single-stranded nucleic acids with four kinds of dNTP and primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, in a buffer solution which contains a thermostable DNA polymerase such that the above mentioned primers are annealed to the single-stranded nucleic acids and extension products are synthesized from the primers,

C: separating the primer extension products from the templates on which they are synthesized to produce single-stranded nucleic acids; and

D: repeatedly conducting the above mentioned steps B and C.

In the step A, the target nucleic acid is denatured if necessary to give a single-stranded nucleic acid. The means therefor may be a thermal treatment, a chemical denaturation or an enzymatic treatment. Preferably, it is a thermal

treatment.

In the step B, said single-stranded nucleic acid is made to react with four kinds of dNTP (dATP, dGTP, dCTP and dTTP or dUTP) and primers with regular and inverted directions having complementary base sequences to the target nucleic acid in a buffer solution containing a thermostable DNA polymerase so that said primers are annealed to the single-stranded nucleic acid to conduct a primer extension reaction.

A primer with a regular direction and that with an inverted direction having complementary base sequences to the target nucleic acid are oligonucleotides having a base sequence which is complementary to one of target nucleic acid and is homologous to another. Accordingly, one primer may be complementary to another primer elongate.

Preferred buffer solutions containing a thermostable DNA polymerase are Tris buffers containing divalent cation such as magnesium ion.

An example of the conditions for conducting an elongation reaction by annealing the primer is a method in which a cycle of 98°C/1 second-1 minute and 68°C/1 second-10 minutes is repeated for 30 times.

The step of separating an elongated primer for making a single strand in the step C may be a thermal treatment, a chemical treatment or an enzymatic treatment. Preferably, it is a thermal treatment or an enzymatic treatment using RNase.

In the step D, the above-mentioned steps B and C are repeated. To be more specific, it is preferred that heating and cooling of 98°C/20 seconds and 68°C/30 seconds are repeated at least for 30 cycles.

An amplifying method of the present invention is applicable to a PCR for amplifying a DNA of 20 kb or more (hereinafter, referred to as a long-PCR) as well. In this long-PCR, advantages of both high DNA extension rate of Taq polymerase and high fidelity in DNA synthesis caused by a 3'-5' exonuclease activity of Pfu polymerase are necessary and both enzymes are used after mixing them. In this case, there is a question on a stability when both enzymes are stored in the same container because of the difference between their thermostabilities and that between the compositions of their stored solutions. However, in the DNA polymerase derived from a hyperthermophilic archaeon strain KOD1, a single enzyme exhibits both high DNA extension rate and high fidelity due to its 3',-5' exonuclease activity whereby it is possible that a long-PCR can be conducted by its sole use.

In the present invention, the amplified product produced by the above-mentioned amplification such as a labeled probe is used whereby a target nucleic acid can be detected.

Labeled probe is an oligonucleotide having a base sequence which is complementary to a target nucleic acid and is bonded with a labeled substance or a labeled binding substance.

Examples of the labeled substance are enzymes such as alkaline phosphatase, peroxidase and galactosidase, fluorescent substances and radioactive substances while examples of the labeled binding substances are biotin and digoxigenin. Labeled substance may be bonded via biotin, digoxigenin or avidin.

A method of introducing those labels into a probe is that, during the synthesis of oligonucleotide, dNTP to which those labeled substances or labeled binding substances are bonded is used as one of the components of dNTP whereby a synthesis is conducted.

Examples of detecting a nucleic acid bonded with a labeled probe are conventionally known methods such as a Southern hybridization and a Northern hybridization. In those methods, the fact that a hybrid is formed when single-stranded DNA and RNA are complementary each other is utilized whereby unknown nucleic acid fraction group is subjected to an agarose electrophoresis to separate its size, then the nucleic acid fraction in the gel is subjected, for example, to an alkali treatment, the resulting single strand is transferred to a filter, immobilized and hybridized with a labeled probe.

As to a detection of the label in case an alkaline phosphatase is used as a labeled substance, when a chemoluminescent substrate such as a 1,2-dioxetane compound (PPD) is made to react therewith, only nucleic acid forming a hybrid is illuminated. This is sensitized to an X-ray film whereby the size of the target nucleic acid and its position on electrophoresis can be confirmed.

A reagent kit for nucleic acid amplification according to the present invention contains primers of regular and inverted directions having base sequences complementary to target nucleic acid, four kinds of dNTP, divalent cation, thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and having a 3'-5' exonuclease activity and a buffer solution.

An example of divalent cation is magnesium ion. Its concentration is preferably about 1-3 mM. Examples of the buffer solution are tris buffer (pH 6.5, 75°C) and tricine buffer (pH 6.5, 75°C).

A specific example of the composition is as follows:

20mM Tris-HCl (pH 6.5, 75°C)

10mM KCl

6mM (NH₄)₂SO₄

1-3mM MgCl₂

0.1% Triton X-100

10 µg/ml BSA

20-200 µM dNTPs

0.1pM-1μM primer

0.1-250ng template DNA.

A reagent kit for nucleic acid amplification according to the present invention contains a nucleic acid amplifying reagent comprising primers of regular and inverted directions having base sequences complementary to target nucleic acid, four kinds of dNTP, divalent cation, thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and having a 3'-5' exonuclease activity and a buffer solution for amplification, a target nucleic acid probe and a buffer for detection. The buffer for detection is that the detecting reagent varies depending upon the label. For example, it includes a color reagent or a luminous reagent.

KOD1 which is a kind of hyperthermophilic archaeon used in the present invention is a strain isolated from a solfatara at a wharf on Kodakara Island, Kagoshima.

Mycological properties of said strain are as follows.

Shape of cells: coccus, diplococcus; having flagella.

Temperature range for the growth: 65-100°C

Optimum temperature for the growth: 95°C

pH range for the growth: 5-9

Optimum pH: 6

Optimum salt concentration: 2-3%

Auxotrophy: heterotrophic

Oxygen demand: aerophobic

Cell membrane lipids: ether type

GC content of DNA: 38%

The hyperthermophilic archaeon strain KOD1 was a coccus having a diameter of about 1 μm and had plural polar flagella. From the mycological properties of the strain, its close relationship with Pfu DNA polymerase-productive bacterium (*Pyrococcus furiosus*) and with Tli (Vent) DNA polymerase-productive bacterium (*Thermococcus litoralis*) was suggested.

Cloning of the thermostable DNA polymerase gene of the present invention is carried out as follows.

The cloning method is that a primer is designed and synthesized depending upon an amino acid sequence in a conserved region of Pfu DNA polymerase (Nucleic Acids Research, 1993, vol.21, No.2, 259-265).

First, a PCR is conducted using the above-prepared primers (e.g., SEQ ID Nos.7 and 8) taking chromosomal DNA of the hyperthermophilic archaeon strain KOD1 as a template to amplify the DNA fragment. The DNA sequence (e.g., SEQ ID No.9) of the amplified fragment is determined and, after confirming that the originally set amino acid sequence is coded for, a Southern hybridization is conducted to the cleaved product of the chromosomal DNA with a restriction enzyme using said fragment as a probe. It is preferred that the approximate size of the fragment containing the aimed DNA polymerase gene is limited to about 4-7 Kbp.

Then DNA fragment of about 4-7 Kbp is recovered from the gel, a DNA library is prepared by *Escherichia coli* using said fragment and a colony hybridization is carried out using the above-mentioned PCR-amplified DNA fragment (e.g., SEQ ID No.9) to collect a clone strain.

The DNA polymerase gene of the strain KOD1 cloned in the present invention is composed of 5010 bases (estimated numbers of amino acids: 1670) (SEQ ID No.5).

Upon comparison with other DNA polymerases, there is a conserved region of αDNA polymerase which is an eukaryote type (Regions 1-5) in the gene of the present invention. In addition, there are EXO 1,2,3 which are 3'→5' exonuclease motives at the N terminal of said gene. In the conserved regions (Regions 1, 2) of the thermostable DNA polymerase gene derived from the hyperthermophilic archaeon strain KOD1, each of the intervening sequences is present and they are connected in a form where the open reading frame (ORF) is conserved.

When the thermostable DNA polymerase gene of the hyperthermophilic archaeon strain KOD1 is compared with Pfu DNA polymerase gene derived from *Pyrococcus furiosus* (Japanese Laid-Open Patent Publication Hei-05/328969) and with Tli (Vent) DNA polymerase gene derived from *Thermococcus litoralis* (Japanese Laid-Open Patent Publication He-06/7160) which are known enzymes, intervening sequence is present in the gene of the strain KOD1 of the present invention while there is no intervening sequence in the gene of the above-mentioned Pfu DNA polymerase and, in the Tli DNA polymerase gene, there are two kinds of intervening sequences but they are present within Regions 2 and 3 which are conserved regions and that greatly differs from the location where the intervening sequence in the thermostable DNA polymerase gene of KOD1 strain of the present invention exists (see Fig. 7).

The gene of the present invention is a DNA which codes for the DNA polymerase derived from the hyperthermophilic archaeon strain KOD1. An example of said DNA contains a base sequence which codes for the amino acid sequence mentioned in SEQ ID No. 1 or 5. Further, such a DNA contains a base sequence mentioned in SEQ ID No. 5 or 6 or a part thereof.

In order to express the thermos table DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 of the present invention in *Escherichia coli*, the intervening sequences of 1374-2453 bp and 2708-4316 bp in the base sequence shown by SEQ ID No.5 are removed by means of a PCR gene fusion to construct a DNA polymerase gene

of a complete form. To be specific, a PCR is conducted on a cloned gene containing the intervening sequence by a combination of three pairs of primers to amplify the three fragments which are divided by the intervening sequence. In designing the primers used here, a part of the fragment which is to be bonded to its terminal is contained in its 5'-end. Then a PCR is conducted using the fragments to be bonded utilizing the duplicated sequence of the terminal whereby each of the fragments is bonded. Further PCR is conducted by the same manner using the resulting two kinds of fragments to give a DNA polymerase gene in a complete form containing no DNA polymerase gene derived from the strain KOD1 containing no intervening sequence.

Any vector may be used in the present invention so far as it makes cloning and expression of the thermostable DNA polymerase derived from KOD1 possible, e.g. a phage or plasmid. An example of the plasmid is a plasmid vector wherein an expression induced by T7 promoter is possible such as pET-8c. Other examples of the plasmid are pUC19, pBR322, pBluescript, pSP73, pGW7, pET3A and pET11C and so on. Examples of the phage are lambda gt11, lambda DASH and lambda ZapII and so on.

Examples of the host cell used in the present invention are Escherichia coli and yeasts. Examples of Escherichia coli are JM109, 101, XL1, PR1 and BL21(DE3)pysS and so on.

In the present invention, the gene coding for the thermostable DNA polymerase derived from the above-mentioned KOD1 is inserted into the above-mentioned vector to give a recombinant vector and the host cell is subjected to a transformation using said recombinant vector.

In the producing method of the present invention, the above-mentioned recombinant host cell is cultured whereby the thermostable DNA polymerase gene derived from the strain KOD1 is induced and expressed. The culture medium used for the culture of the recombinant host cell and the conditions therefor follow the conventional methods.

In a specific example, Escherichia coli which is transformed by pET-8c plasmid containing a DNA polymerase gene in a complete form containing no intervening sequence derived from the strain KOD1 is cultured, for example, in a TB medium whereby an induction treatment is conducted. It is preferred that the induction treatment of T7 promoter is carried out by addition of isopropylthio- β -D-galactoside.

The purifying method of the present invention includes, after culturing the recombinant host cells, a step wherein (a) recombinant host cells are collected, disintegrated and the cell extract is prepared and a step wherein (b) impure protein derived from the host cells is removed.

The thermostable DNA polymerase which is produced from the recombinant host cells is separated and recovered from the culture liquid by means of centrifugation or the like after culturing the host bacterial cells in a medium followed by inducing. After said bacterial cells are resuspended in a buffer, they are disintegrated by means of ultrasonic treatment, Dyno mill, French press, etc. Then a thermal treatment is conducted and the heat stable DNA polymerase is recovered from the supernatant fluid. In disintegrating the bacterial cells, ultrasonic treatment, Dyno mill and French press method are preferred.

A thermal treatment is preferred as one of the steps for removing the impure protein derived from the host cells. The condition for the thermal treatment is at 70°C or higher or, preferably, at 90° or higher. Other means for removing the impure protein are various chromatographic techniques.

Molecular weight of the thermostable DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 obtained as such is about 90 KDa (cf. Fig. 5).

When a polymerase chain reaction is conducted using said thermostable DNA polymerase, a sufficient amplification of the aimed DNA fragments is confirmed (cf. Fig. 6).

Now the present invention will be illustrated by referring partly to the drawings wherein:

Fig. 1 is a photographic picture of electrophoresis as a substitute for a drawing and shows the result of the measurement of the DNA extension rate of the KOD polymerase;

Fig. 2 is a photographic picture of electrophoresis as a substitute for a drawing and shows the comparison of the DNA extension rate of various thermostable DNA polymerases in which Fig. 2a shows the cases of KOD polymerase and Pfu polymerase while Fig. 2b shows the cases of Deep Vent polymerase and Taq polymerase;

Fig. 3 is a photographic picture of electrophoresis as a substitute for a drawing and shows the comparison of the PCR due to the difference in the reaction time of various thermostable DNA polymerase;

Fig. 4 shows the constructive charts of the recombinant expression vector;

Fig. 5 is a photographic picture of electrophoresis as a substitute for a drawing and shows the result of the measurement of molecular weight of the thermostable DNA polymerase derived from KOD1;

Fig. 6 is a photographic picture of electrophoresis as a substitute for a drawing and shows the result of the PCR by the thermostable DNA polymerase derived from KOD1; and

Fig. 7 shows drawings which show a comparison of the DNA polymerase gene derived from the hyperthermophilic archaeon strain KOD1 with the thermostable DNA polymerase gene derived from Pyrococcus furiosus and that derived from Thermococcus litoralis which are thought to be similar bacteria.

Example 1.

Cloning of DNA Polymerase Gene Derived from hyperthermophilic archaeon strain KOD1

The hyperthermophilic archaeon strain KOD1 isolated in Kodakara Island, Kagoshima was cultured at 95°C and then the bacterial cells were recovered. Chromosomal DNA of the hyperthermophilic archaeon strain KOD1 was prepared by a conventional method from the resulting bacterial cells.

Two kinds of primers (5'-GGATTAGTATAGTGCCAATGGAAGGCGAC-3' [SEQ ID No.7] and 5'-GAGGGCGAAGTT-TATTCCGAGCTT-3' [SEQ ID No.8]) were synthesized based upon the amino acid sequence at the conserved region of the DNA polymerase (Pfu polymerase) derived from *Pyrococcus furiosus*. A PCR was carried out using those two primers where the prepared chromosomal DNA was used as a template.

After the base sequence (SEQ ID No.9) of the PCR-amplified DNA fragment was determined and the amino acid sequence (SEQ ID No.10) was determined, a Southern hybridization was conducted using said amplified DNA fragment to the product of the strain KOD1 chromosomal DNA treated with a restriction enzyme whereby the size of the fragment coding for the DNA polymerase was calculated (about 4-7 Kbp). Further, the DNA fragment of this size was recovered from agarose gel, inserted into a plasmid pBS (manufactured by Stratagene) and *Escherichia coli* (*E. coli* JM 109) was transformed by this mixture to prepare a library.

A colony hybridization was conducted using a probe (SEQ ID No.9) used for the Southern hybridization to obtain a clone strain (*E. coli* JM109/pBSKOD1) which is thought to contain the DNA polymerase gene derived from strain KOD1.

Example 2.

Determination of Base Sequence of the cloned Fragment

A plasmid pBSKOD1 was recovered from the clone strain *E. coli* JM109/pBSKOD1 obtained in Example 1 and its base sequence (SEQ ID No.5) was determined by a conventional method. Further, the amino acid sequence was derived from the determined base sequence. The DNA polymerase gene derived from KOD1 strain comprised 5010 bases wherein 1670 amino acids were coded.

Example 3.

Construction of Recombinant Expression Vector

In order to prepare a complete polymerase gene, the intervening sequence parts at two places (1374-2453 bp and 2708-4316 bp) were removed by a PCR fusion method. In the PCR fusion method, three pairs of primers (SEQ ID Nos.11-16) were combined using a primer recovered from the clone strain as a template and a PCR was conducted for each of them to amplify three fragments wherefrom the intervening sequences were removed. At that time, the primer used for the PCR was designed in such a manner that the side which binds to another fragment has the same sequence as the binding partner has. In addition, a design was conducted in such a manner that different restriction enzyme sites (EcoRV at the N-terminus while BamHI at the C-terminus) were created at both ends.

After that, among the PCR-amplified fragments, that which is located at the central part of the structure and that which is located at the N-terminal side are mixed and a PCR was conducted using each of the fragments as a primer. At the same time, the fragment located at the central part of the structure and that located at the C-terminal side are mixed and a PCR was conducted using each of the fragments as a primer. Two kinds of fragments obtained as such were subjected to a PCR once again to give gene fragments in a complete form having no intervening sequence, having EcoRV and BamHI sites at the N- and C-termini, respectively and coding for the DNA polymerase derived from strain KOD1.

Further, said gene was subcloned using an expression vector which can be induced by T7 promoter, an NcoI/BamHI site of pET-8c and the previously-created restriction enzyme site to give a recombinant expression vector (pET-pol).

Example 4.

Expression and Purification of DNA Polymerase Derived from KOD1

Escherichia coli (BL21(DE3)) was transformed using a recombinant expression vector (pET-pol) obtained in Example 3, the resulting transformant was cultured in a TB medium (mentioned in Molecular Cloning, p.A.2, 1989) and, one hour before collecting the bacterial cells, an induction treatment of T7 promoter was conducted by addition of isopropylthio-β-D-galactopyrenoside. Bacterial cells were recovered from the cultured liquid by means of centrifugation. They

were resuspended in a buffer and disintegrated by an ultrasonic treatment to give a cell extract. In order to remove the impure protein derived from the host cells, the disintegrated cell solution was treated at 94°C for 20 minutes whereby the impure protein derived from the host cells was isolated by centrifugation to give a thermostable DNA polymerase derived from strain KOD1.

The *Escherichia coli* BL21 (DE3) pER-pol was deposited on April 22, 1996 under the Budapest Treaty at National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology (1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN) in accordance with the Budapest Treaty under the accession number FERM BP-5513.

Example 5.

Purification of Thermostable DNA Polymerase Derived from KOD1

Molecular weight of the thermostable DNA polymerase derived from KOD1 obtained in Example 4 was calculated by means of an SDS-PAGE method whereby it was found to be about 86-92 kDa (Fig. 5). Further, a PCR was conducted using the thermostable DNA polymerase derived from KOD1 obtained in Example 4 and the known template primer whereupon a DNA fragment which was to be a target was confirmed (Fig. 6) by the same manner as in the case where the thermostable DNA polymerase derived from *Thermococcus litoralis* was used and a high thermostable DNA polymerase activity was confirmed.

Comparative Example 1.

Comparison with the Thermostable DNA Polymerase Gene Derived from *Pyrococcus furiosus* or from *Thermococcus litoralis* which are to be Similar to the Hyperthermophilic archaeon strain KOD1 of the Present Invention.

Amino acid sequences were estimated from the DNA sequences of the DNA polymerase gene derived from the hyperthermophilic archaeon strain KOD1 of the present invention (SEQ ID No.6), the thermostable DNA polymerase gene derived from *Pyrococcus furiosus* (Japanese Laid-Open Patent Publication Hei-5/328969) and the thermostable DNA polymerase gene derived from *Thermococcus litoralis* (Japanese Laid-Open Patent Publication Hei-6/7160) and were compared and investigated.

In the DNA polymerase derived from KOD1 of the present invention, there were regions 1-5 which were the conserved regions of α DNA polymerase of an eukaryotic type. Further, there were EXO1, 2 and 3 which were 3'→5' exonuclease motives at the N-terminal side. However, in each of the Region 1 and Region 2 which were the α DNA polymerase conserved regions, there were intervening sequences IVS-A and IVS-B (refer to Fig. 7).

On the other hand, in Pfu polymerase which is a thermostable DNA polymerase derived from *Pyrococcus furiosus*, there was no intervening sequence. In the case of Vent polymerase which is a thermostable DNA polymerase derived from *Thermococcus litoralis*, there were the intervening sequences (IVS1 and IVS2) in the α DNA polymerase conserved regions (Region 2 and Region 3) (see Fig.7).

Example 6.

Measurement of DNA Extension Rate of the DNA Polymerase Derived from Hyperthermophilic archaeon strain KOD1

DNA prepared by annealing the M13mp18DNA with M13P7 primer having a base sequence as mentioned in SEQ ID No.2 was used as a substrate and the rate of synthesizing the DNA in a reaction buffer solution [20 mM Tris-HCl (pH 7.5 at 75°C), 10 mM KCl, 6 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1% Triton X-100 and 10 μ g/ml nuclease-free BSA] containing the DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 manufactured in Examples 1-5 was investigated for the reaction time of 20, 40, 60, 80 and 100 seconds (Fig. 1) or 40, 60, 80 and 100 seconds (Fig. 2). The results are given in Fig. 1 and in Fig. 2.

A part of the DNA sample during the elongation reaction was taken out for each reaction time and was added to a reaction stopping solution (60 mM EDTA, 60 μ M NaOH, 0.1% BPB and 30% glycerol) in the same amount.

The DNA samples obtained in the above process were separated and analyzed by means of an alkaline agarose electrophoresis and the size of the synthesized DNA was checked.

1, 2, 3, 4 and 5 in Fig. 1 show the results of the reactions for 0.3 minute (20 seconds), 0.7 minute (40 seconds), 1 minute (60 seconds), 1.3 minutes (80 seconds) and 1.7 minutes (100 seconds), respectively. It is apparent from Fig. 1 that the DNA extension rate of the DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 was 105 bases/second.

1, 2, 3 and 4 in Fig. 2 show the results of the reaction for 0.7 minute (40 seconds), 1 minute (60 seconds), 1.3 minutes (80 seconds) and 1.7 minutes (100 seconds), respectively. It is apparent from Fig. 2 that the DNA extension rate

of the DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 was 138 bases/second.

On the other hand, the DNA synthesizing rate of each of Pfu polymerase (Stratgene), Deep Vent polymerase (New England Biolabs) and Taq polymerase (Takara Shuzo) was measured by the same manner in each of the buffers therefor (Fig. 2a and Fig. 2b). The DNA extension rates of those DNA polymerases were 24.8 bases/second for Pfu polymerase, 23.2 bases/second for Deep Vent polymerase and 61.0 bases/second for Taq polymerase.

From the above results, it was suggested that the DNA extension rate of the DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 was about six-fold of those of Pfu polymerase and Deep Vent polymerase and about two-fold of that of Taq polymerase.

Example 7.

Measurement of Fidelity of the DNA Polymerase Derived from the Hyperthermophilic archaeon strain KOD1 in the Reaction for the Synthesis of DNA

The error rate in the DNA synthesis was measured by the method of Kunkel (Kunkel, 1985, Journal of Biological Chemistry, 260, 5787-5796). In this method, a DNA synthesis reaction was conducted using a DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 manufactured in Examples 1-5 using an M13mp18DNA having a gap at a lacZ part containing a part of the genes coding for β -galactosidase as a substrate and transfected to *E. coli* JM109 in an NZY medium containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside and isopropyl-thio- β -D-galactoside using an M13mp18DNA in which lacZ part was double-stranded.

When β -galactosidase wherein a function is lost or lowered was expressed due to a reading error or a frame shift during the synthetic reaction of DNA, it is not possible to utilize 5-bromo-4-chloro-3-indolyl- β -D-galactoside whereupon the color of plaque becomes colorless or light blue. On the other hand, when there is no error in the synthesized DNA and a complete β -galactosidase was expressed, plaque becomes blue. The rate of induction of error was measured in the DNA synthesis from the rate of the sum of colorless and light blue plaque to the total plaque.

The rate of induction of error in the DNA synthesis was also measured for Pfu polymerase (Stratgene), Taq polymerase (Takara Shuzo) and delta Tth polymerase (Toyobo) which were made to react by the same manner.

Further, the rate of induction of error in the DNA synthesis was also measured for a mixture of Taq polymerase and Pfu polymerase. The results are given in Table 1.

Table 1

Measurement of Fidelity in the Reaction of DNA Synthesis of DNA Polymerase Derived from Hyperthermophilic archaeon strain KOD1					
Enzyme	Light Blue	White	Mutant	Total	Mutant Frequency(10^{-4})
KOD1 pol.	12	11	23	6619	37.7
Pfu	15	15	30	7691	39.0
Taq	30	24	54	4141	130
Δ Tth	70	45	115	7375	156
Taq/Pfu(20:1)	10	20	30	4238	63.7
Taq/Pfu(50:1)	10	13	23	4489	53.5

It is apparent from Table 1 that the fidelity of the DNA polymerase derived from hyperthermophilic archaeon strain KOD1 in the DNA synthesis reaction is suggested to be superior to Taq polymerase and same as Pfu polymerase. In addition, a mixture of Taq polymerase and Pfu polymerase exhibits a medium fidelity that it is superior to Taq polymerase and inferior to Pfu polymerase.

Example 8.

Comparison in PCR of Various Thermostable DNA Polymerases by the Difference in the Reaction Time

lambda-DNA (3 μ g) was used as a target nucleic acid; oligo-nucleotides having a sequence as mentioned in SEQ ID Nos. 3 and 4 were used as primers; and a buffer containing 20 mM Tris-HCl (pH 7.5 at 75°C), 10 mM KCl, 6 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 0.1% Triton X-100, 10 μ g/ml BSA and 200 μ M dNTPs was used as a buffer. DNA polymerase

derived from hyperthermophilic archaeon strain KOD1 (KOD polymerase), Taq polymerase which is widely used for PCR and Pfu polymerase which exhibits 3'-5' exonuclease activity were also used as the thermostable DNA polymerases. The used titer of each polymerase was 2 units.

5 A PCR amplification reaction was conducted using a DNA Thermal Cycler (Perkin-Elmer) in a schedule wherein a cycle comprising 94°C/20 seconds and 68°C/x second (x: reaction time) was repeated for 30 times. In the case of the DNA polymerase derived from the hyperthermophilic archaeon strain KOD1 (KOD polymerase), amplification of the target DNA was confirmed by conducting 30 cycles of 94°C/20 seconds-68°C/1 second while, in the case of Taq polymerase, amplification of DNA was first confirmed by conducting 30 cycles of 94°C/20 seconds-68°C/10 seconds. In the case of Pfu polymerase, amplification of DNA was at least confirmed by conducting 30 cycles of 94°C/20 seconds-68°C/1 minute. The results are given in Fig. 3.

10 In the present invention, it is possible to amplify the DNA with a high fidelity within a short reaction time when a DNA polymerase derived from hyperthermophilic archaeon strain KOD1 which is a thermostable DNA polymerase having at least 30 bases/second of DNA extension rate and having a 3'-5' exonuclease activity. When this method is carried out, it is possible to improve the simplicity and convenience. In addition, when such kind of thermostable DNA polymerase having both high extension rate (at least 30 bases/second) which has not been available yet and 3'-5' exonuclease activity is used, it is possible to shorten the time for the primer extension reaction and to amplify the relatively big product with a high fidelity.

SEQUENCE LISTING

SEQ ID No.1

Length: 774 base pairs

Type: amino acid

Topology: linear

Type: protein

Sequence Description:

Met Ile Leu Asp Thr Asp Tyr Ile Thr Glu Asp Gly Lys Pro Val Ile

1 5 10 15

Arg Ile Phe Lys Lys Glu Asn Gly Glu Phe Lys Ile Glu Tyr Asp Arg

20 25 30

Thr Phe Glu Pro Tyr Phe Tyr Ala Leu Leu Lys Asp Asp Ser Ala Ile

35 40 45

Glu Glu Val Lys Lys Ile Thr Ala Glu Arg His Gly Thr Val Val Thr

50 55 60

Val Lys Arg Val Glu Lys Val Gln Lys Lys Phe Leu Gly Arg Pro Val

65 70 75 80

Glu Val Trp Lys Leu Tyr Phe Thr His Pro Gln Asp Val Pro Ala Ile

85 90 95

Arg Asp Lys Ile Arg Glu His Gly Ala Val Ile Asp Phe Tyr Glu Tyr

100 105 110

Asp Ile Pro Phe Ala Lys Arg Tyr Leu Ile Asp Lys Gly Leu Val Pro

115 120 125

Met Glu Gly Asp Glu Glu Leu Lys Met Leu Ala Phe Asp Ile Gln Thr

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	Pro	Asn	Lys	Pro	Asp	Glu	Lys	Glu	Leu	Ala	Arg	Arg	Arg	Gln	Ser	Tyr
	370						375						380			
5	Glu	Gly	Gly	Tyr	Val	Lys	Glu	Pro	Glu	Arg	Gly	Leu	Trp	Glu	Asn	Ile
	385					390					395				400	
10	Val	Tyr	Leu	Asp	Phe	Arg	Ser	Leu	Tyr	Pro	Ser	Ile	Ile	Ile	Thr	His
					405					410					415	
	Asn	Val	Ser	Pro	Asp	Thr	Leu	Asn	Arg	Glu	Gly	Cys	Lys	Glu	Tyr	Asp
15					420				425					430		
	Val	Ala	Pro	Gln	Val	Gly	His	Arg	Phe	Cys	Lys	Asp	Phe	Pro	Gly	Phe
		435						440						445		
20	Ile	Pro	Ser	Leu	Leu	Gly	Asp	Leu	Leu	Glu	Glu	Arg	Gln	Lys	Ile	Lys
	450						455					460				
	Lys	Lys	Met	Lys	Ala	Thr	Ile	Asp	Pro	Ile	Glu	Arg	Lys	Leu	Leu	Asp
25	465					470					475				480	
	Tyr	Arg	Gln	Arg	Ala	Ile	Lys	Ile	Leu	Ala	Asn	Ser	Tyr	Tyr	Gly	Tyr
					485					490					495	
30	Tyr	Gly	Tyr	Ala	Arg	Ala	Arg	Trp	Tyr	Cys	Lys	Glu	Cys	Ala	Glu	Ser
				500					505					510		
35	Val	Thr	Ala	Trp	Gly	Arg	Glu	Tyr	Ile	Thr	Met	Thr	Ile	Lys	Glu	Ile
		515							520					525		
	Glu	Glu	Lys	Tyr	Gly	Phe	Lys	Val	Ile	Tyr	Ser	Asp	Thr	Asp	Gly	Phe
40		530					535						540			
	Phe	Ala	Thr	Ile	Pro	Gly	Ala	Asp	Ala	Glu	Thr	Val	Lys	Lys	Lys	Ala
	545					550					555				560	
45	Met	Glu	Phe	Leu	Asn	Tyr	Ile	Asn	Ala	Lys	Leu	Pro	Gly	Ala	Leu	Glu
					565					570					575	
	Leu	Glu	Tyr	Glu	Gly	Phe	Tyr	Lys	Arg	Gly	Phe	Phe	Val	Thr	Lys	Lys
50					580				585						590	
	Lys	Tyr	Ala	Val	Ile	Asp	Glu	Glu	Gly	Lys	Ile	Thr	Thr	Arg	Gly	Leu

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	130	135	140	
5	Leu Tyr His Glu Gly Glu Glu Phe Ala Glu Gly Pro Ile Leu Met Ile			
	145	150	155	160
	Ser Tyr Ala Asp Glu Glu Gly Ala Arg Val Ile Thr Trp Lys Asn Val			
10	165	170	175	
	Asp Leu Pro Tyr Val Asp Val Val Ser Thr Glu Arg Glu Met Ile Lys			
	180	185	190	
15	Arg Phe Leu Arg Val Val Lys Glu Lys Asp Pro Asp Val Leu Ile Thr			
	195	200	205	
	Tyr Asn Gly Asp Asn Phe Asp Phe Ala Tyr Leu Lys Lys Arg Cys Glu			
20	210	215	220	
	Lys Leu Gly Ile Asn Phe Ala Leu Gly Arg Asp Gly Ser Glu Pro Lys			
25	225	230	235	240
	Ile Gln Arg Met Gly Asp Arg Phe Ala Val Glu Val Lys Gly Arg Ile			
	245	250	255	
30	His Phe Asp Leu Tyr Pro Val Ile Arg Arg Thr Ile Asn Leu Pro Thr			
	260	265	270	
	Tyr Thr Leu Glu Ala Val Tyr Glu Ala Val Phe Gly Gln Pro Lys Glu			
35	275	280	285	
	Lys Val Tyr Ala Glu Glu Ile Thr Pro Ala Trp Glu Thr Gly Glu Asn			
	290	295	300	
40	Leu Glu Arg Val Ala Arg Tyr Ser Met Glu Asp Ala Lys Val Thr Tyr			
	305	310	315	320
	Glu Leu Gly Lys Glu Phe Leu Pro Met Glu Ala Gln Leu Ser Arg Leu			
45	325	330	335	
	Ile Gly Gln Ser Leu Trp Asp Val Ser Arg Ser Ser Thr Gly Asn Leu			
	340	345	350	
50	Val Glu Trp Phe Leu Leu Arg Lys Ala Tyr Glu Arg Asn Glu Leu Ala			
	355	360	365	

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595 600 605
 Glu Ile Val Arg Arg Asp Trp Ser Glu Ile Ala Lys Glu Thr Gln Ala
 5 610 615 620
 Arg Val Leu Glu Ala Leu Leu Lys Asp Gly Asp Val Glu Lys Ala Val
 625 630 635 640
 10 Arg Ile Val Lys Glu Val Thr Glu Lys Leu Ser Lys Tyr Glu Val Pro
 645 650 655
 15 Pro Glu Lys Leu Val Ile His Glu Gln Ile Thr Arg Asp Leu Lys Asp
 660 665 670
 Tyr Lys Ala Thr Gly Pro His Val Ala Val Ala Lys Arg Leu Ala Ala
 20 675 680 685
 Arg Gly Val Lys Ile Arg Pro Gly Thr Val Ile Ser Tyr Ile Val Leu
 690 695 700
 25 Lys Gly Ser Gly Arg Ile Gly Asp Arg Ala Ile Pro Phe Asp Glu Phe
 705 710 715 720
 Asp Pro Thr Lys His Lys Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn Gln
 30 725 730 735
 Val Leu Pro Ala Val Glu Arg Ile Leu Arg Ala Phe Gly Tyr Arg Lys
 740 745 750
 35 Glu Asp Leu Arg Tyr Gln Lys Thr Arg Gln Val Gly Leu Ser Ala Trp
 755 760 765
 40 Leu Lys Pro Lys Gly Thr
 770
 45
 50
 55

SEQ ID No.2

Length: 24 base pairs

Type: nucleic acid

Topology: linear

Type: synthetic DNA

Sequence Description:

CGCCAGGGTT TTCCAGTCA CGAC

24

SEQ ID No.3

Length: 20 base pairs

Type: nucleic acid

Topology: linear

Molecular Type: synthetic DNA

Sequence Description:

GGGCGGCGAC CTCGCGGGTT

20

SEQ ID No.4

Length: 24 base pairs

Type: nucleic acid

Topology: linear

Molecular Type: synthetic DNA

Sequence Description:

GCCCATAATA ATCTGCCGGT CAAT

24

SEQ ID No.5

Length: 5342 base pairs

Type: nucleic acid (DNA)

Strandedness: double

Topology: linear

Molecular Type: cDNA

Source: hyperthermophilic archaeon

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Strain name: KOD1

Characteristics: { 156-5165 P CDS
1374-2453 intervening sequence
2708-4316 intervening sequence

Sequence Description:

	GCTTGAGGGC CTGCGGTAT GGGACGTTGC AGTTTGGCC TACTCAAAGA TGCCGGTTTT	60
10	ATAACGGAGA AAAATGGGA GCTATTACGA TCTCTCCTTG ATGTGGGTT TACAATAAG	120
	CCTGGATTGT TCTACAAGAT TATGGGGGAT GAAAG ATG ATC CTC GAC ACT GAC	173
	Met Ile Leu Asp Thr Asp	
15	1 5	
	TAC ATA ACC GAG GAT GGA AAG CCT GTC ATA AGA ATT TTC AAG AAG GAA	221
	Tyr Ile Thr Glu Asp Gly Lys Pro Val Ile Arg Ile Phe Lys Lys Glu	
20	10 15 20	
	AAC GGC GAG TTT AAG ATT GAG TAC GAC CGG ACT TTT GAA CCC TAC TTC	269
	Asn Gly Glu Phe Lys Ile Glu Tyr Asp Arg Thr Phe Glu Pro Tyr Phe	
25	25 30 35	
	TAC GCC CTC CTG AAG GAC GAT TCT GCC ATT GAG GAA GTC AAG AAG ATA	317
	Tyr Ala Leu Leu Lys Asp Asp Ser Ala Ile Glu Glu Val Lys Lys Ile	
30	40 45 50	
	ACC GCC GAG AGG CAC GGG ACG GTT GTA ACG GTT AAG CGG GTT GAA AAG	365
	Thr Ala Glu Arg His Gly Thr Val Val Thr Val Lys Arg Val Glu Lys	
35	55 60 65 70	
	GTT CAG AAG AAG TTC CTC GGG AGA CCA GTT GAG GTC TGG AAA CTC TAC	413
	Val Gln Lys Lys Phe Leu Gly Arg Pro Val Glu Val Trp Lys Leu Tyr	
40	75 80 85	
	TTT ACT CAT CCG CAG GAC GTC CCA GCG ATA AGG GAC AAG ATA CGA GAG	461
	Phe Thr His Pro Gln Asp Val Pro Ala Ile Arg Asp Lys Ile Arg Glu	
45	90 95 100	
	CAT GGA GCA GTT ATT GAC ATC TAC GAG TAC GAC ATA CCC TTC GCC AAG	509
	His Gly Ala Val Ile Asp Ile Tyr Glu Tyr Asp Ile Pro Phe Ala Lys	
50	105 110 115	
55		

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5	CGC TAC CTC ATA GAC AAG GGA TTA GTG CCA ATG GAA GGC GAC GAG GAG	557
	Arg Tyr Leu Ile Asp Lys Gly Leu Val Pro Met Glu Gly Asp Glu Glu	
	120 125 130	
10	CTG AAA ATG CTC GCC TTC GAC ATT CAA ACT CTC TAC CAT GAG GGC GAG	605
	Leu Lys Met Leu Ala Phe Asp Ile Gln Thr Leu Tyr His Glu Gly Glu	
	135 140 145 150	
15	GAG TTC GCC GAG GGG CCA ATC CTT ATG ATA AGC TAC GCC GAC GAG GAA	653
	Glu Phe Ala Glu Gly Pro Ile Leu Met Ile Ser Tyr Ala Asp Glu Glu	
	155 160 165	
20	GGG GCC AGG GTG ATA ACT TGG AAG AAC GTG GAT CTC CCC TAC GTT GAC	701
	Gly Ala Arg Val Ile Thr Trp Lys Asn Val Asp Leu Pro Tyr Val Asp	
	170 175 180	
25	GTC GTC TCG ACG GAG AGG GAG ATG ATA AAG CGC TTC CTC CGT GTT GTG	749
	Val Val Ser Thr Glu Arg Glu Met Ile Lys Arg Phe Leu Arg Val Val	
	185 190 195	
30	AAG GAG AAA GAC CCG GAC GTT CTC ATA ACC TAC AAC GGC GAC AAC TTC	797
	Lys Glu Lys Asp Pro Asp Val Leu Ile Thr Tyr Asn Gly Asp Asn Phe	
	200 205 210	
35	GAC TTC GCC TAT CTG AAA AAG CGC TGT GAA AAG CTC GGA ATA AAC TTC	845
	Asp Phe Ala Tyr Leu Lys Lys Arg Cys Glu Lys Leu Gly Ile Asn Phe	
	215 220 225 230	
40	GCC CTC GGA AGG GAT GGA AGC GAG CCG AAG ATT CAG AGG ATG GGC GAC	893
	Ala Leu Gly Arg Asp Gly Ser Glu Pro Lys Ile Gln Arg Met Gly Asp	
	235 240 245	
45	AGG TTT GCC GTC GAA GTG AAG GGA CGG ATA CAC TTC GAT CTC TAT CCT	941
	Arg Phe Ala Val Glu Val Lys Gly Arg Ile His Phe Asp Leu Tyr Pro	
	250 255 260	
50	GTG ATA AGA CGG ACG ATA AAC CTG CCC ACA TAC ACG CTT GAG GCC GTT	989
	Val Ile Arg Arg Thr Ile Asn Leu Pro Thr Tyr Thr Leu Glu Ala Val	

55

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	265	270	275	
5	TAT GAA GCC GTC TTC GGT CAG CCG AAG GAG AAG GTT TAC GCT GAG GAA	1037		
	Tyr Glu Ala Val Phe Gly Gln Pro Lys Glu Lys Val Tyr Ala Glu Glu			
	280	285	290	
10	ATA ACA CCA GCC TGG GAA ACC GGC GAG AAC CTT GAG AGA GTC GCC CGC	1085		
	Ile Thr Pro Ala Trp Glu Thr Gly Glu Asn Leu Glu Arg Val Ala Arg			
	295	300	305	310
15	TAC TCG ATG GAA GAT GCG AAG GTC ACA TAC GAG CTT GGG AAG GAG TTC	1133		
	Tyr Ser Met Glu Asp Ala Lys Val Thr Tyr Glu Leu Gly Lys Glu Phe			
	315	320	325	
20	CTT CCG ATG GAG GCC CAG CTT TCT CGC TTA ATC GGC CAG TCC CTC TGG	1181		
	Leu Pro Met Glu Ala Gln Leu Ser Arg Leu Ile Gly Gln Ser Leu Trp			
	330	335	340	
25	GAC GTC TCC CGC TCC AGC ACT GGC AAC CTC GTT GAG TGG TTC CTC CTC	1229		
	Asp Val Ser Arg Ser Ser Thr Gly Asn Leu Val Glu Trp Phe Leu Leu			
	345	350	355	
30	AGG AAG GCC TAT GAG AGG AAT GAG CTG GCC CCG AAC AAG CCC GAT GAA	1277		
	Arg Lys Ala Tyr Glu Arg Asn Glu Leu Ala Pro Asn Lys Pro Asp Glu			
	360	365	370	
35	AAG GAG CTG GCC AGA AGA CGG CAG AGC TAT GAA GGA GGC TAT GTA AAA	1325		
	Lys Glu Leu Ala Arg Arg Arg Gln Ser Tyr Glu Gly Gly Tyr Val Lys			
	375	380	385	390
40	GAG CCC GAG AGA GGG TTG TGG GAG AAC ATA GTG TAC CTA GAT TTT AGA	1373		
	Glu Pro Glu Arg Gly Leu Trp Glu Asn Ile Val Tyr Leu Asp Phe Arg			
	395	400	405	
45	TGC CAT CCA GCC GAT ACG AAG GTT GTC GTC AAG GGG AAG GGG ATT ATA	1421		
	Cys His Pro Ala Asp Thr Lys Val Val Val Lys Gly Lys Gly Ile Ile			
	410	415	420	
50	AAC ATC AGC GAG GTT CAG GAA GGT GAC TAT GTC CTT GGG ATT GAC GGC	1465		
55				

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	ACT CCA AGC ATC TCG GAG AAG AAA GGA ACT AAC GCA GTA ACA CTC AAA	1949
5	Thr Pro Ser Ile Ser Glu Lys Lys Gly Thr Asn Ala Val Thr Leu Lys	
	585 590 595	
	GTT GCG AAG AAG AAT GTT TAT CTT AAA GTC AAG GAA ATT ATG GAC AAC	1997
10	Val Ala Lys Lys Asn Val Tyr Leu Lys Val Lys Glu Ile Met Asp Asn	
	600 605 610	
	ATA GAG TCC CTA CAT GCC CCC TCG GTT CTC AGG GGA TTC TTC GAA GGC	2045
15	Ile Glu Ser Leu His Ala Pro Ser Val Leu Arg Gly Phe Phe Glu Gly	
	615 620 625 630	
	GAC GGT TCA GTA AAC AGG GTT AGG AGG AGT ATT GTT GCA ACC CAG GGT	2093
20	Asp Gly Ser Val Asn Arg Val Arg Arg Ser Ile Val Ala Thr Gln Gly	
	635 640 645	
	ACA AAG AAC GAG TGG AAG ATT AAA CTG GTG TCA AAA CTG CTC TCC CAG	2141
25	Thr Lys Asn Glu Trp Lys Ile Lys Leu Val Ser Lys Leu Leu Ser Gln	
	650 655 660	
	CTT GGT ATC CCT CAT CAA ACG TAC ACG TAT CAG TAT CAG GAA AAT GGG	2189
30	Leu Gly Ile Pro His Gln Thr Tyr Thr Tyr Gln Tyr Gln Glu Asn Gly	
	665 670 675	
	AAA GAT CGG AGC AGG TAT ATA CTG GAG ATA ACT GGA AAG GAC GGA TTG	2237
35	Lys Asp Arg Ser Arg Tyr Ile Leu Glu Ile Thr Gly Lys Asp Gly Leu	
	680 685 690	
	ATA CTG TTC CAA ACA CTC ATT GGA TTC ATC AGT GAA AGA AAG AAC GCT	2285
40	Ile Leu Phe Gln Thr Leu Ile Gly Phe Ile Ser Glu Arg Lys Asn Ala	
	695 700 705 710	
	CTG CTT AAT AAG GCA ATA TCT CAG AGG GAA ATG AAC AAC TTG GAA AAC	2333
45	Leu Leu Asn Lys Ala Ile Ser Gln Arg Glu Met Asn Asn Leu Glu Asn	
	715 720 725	
	AAT GGA TTT TAC AGG CTC AGT GAA TTC AAT GTC AGC ACG GAA TAC TAT	2381
50	Asn Gly Phe Tyr Arg Leu Ser Glu Phe Asn Val Ser Thr Glu Tyr Tyr	
55		

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	730	735	740	
5	GAG GGC AAG GTC TAT GAC TTA ACT CTT GAA GGA ACT CCC TAC TAC TTT			2429
	Glu Gly Lys Val Tyr Asp Leu Thr Leu Glu Gly Thr Pro Tyr Tyr Phe			
	745	750	755	
10	GCC AAT GGC ATA TTG ACC CAT AAC TCC CTG TAC CCC TCA ATC ATC ATC			2477
	Ala Asn Gly Ile Leu Thr His Asn Ser Leu Tyr Pro Ser Ile Ile Ile			
	760	765	770	
15	ACC CAC AAC GTC TCG CCG GAT ACG CTC AAC AGA GAA GGA TGC AAG GAA			2525
	Thr His Asn Val Ser Pro Asp Thr Leu Asn Arg Glu Gly Cys Lys Glu			
	775	780	785	790
20	TAT GAC GTT GCC CCA CAG GTC GGC CAC CGC TTC TGC AAG GAC TTC CCA			2573
	Tyr Asp Val Ala Pro Gln Val Gly His Arg Phe Cys Lys Asp Phe Pro			
	795	800	805	
25	GGA TTT ATC CCG AGC CTG CTT GGA GAC CTC CTA GAG GAG AGG CAG AAG			2621
	Gly Phe Ile Pro Ser Leu Leu Gly Asp Leu Leu Glu Glu Arg Gln Lys			
	810	815	820	
30	ATA AAG AAG AAG ATG AAG GCC ACG ATT GAC CCG ATC GAG AGG AAG CTC			2669
	Ile Lys Lys Lys Met Lys Ala Thr Ile Asp Pro Ile Glu Arg Lys Leu			
	825	830	835	
35	CTC GAT TAC AGG CAG AGG GCC ATC AAG ATC CTG GCA AAC AGC ATC CTA			2717
	Leu Asp Tyr Arg Gln Arg Ala Ile Lys Ile Leu Ala Asn Ser Ile Leu			
	840	845	850	
40	CCC GAG GAA TGG CTT CCA GTC CTC GAG GAA GGG GAG GTT CAC TTC GTC			2765
	Pro Glu Glu Trp Leu Pro Val Leu Glu Glu Gly Glu Val His Phe Val			
	855	860	865	870
45	AGG ATT GGA GAG CTC ATA GAC CGG ATG ATG GAG GAA AAT GCT GGG AAA			2813
	Arg Ile Gly Glu Leu Ile Asp Arg Met Met Glu Glu Asn Ala Gly Lys			
	875	880	885	
50	GTA AAG AGA GAG GGC CAG ACG GAA GTG CTT GAG GTC AGT GGG CTT GAA			2861
55				

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	Val Lys Arg Glu Gly Glu Thr Glu Val Leu Glu Val Ser Gly Leu Glu	
	890 895 900	
5	GTC CCG TCC TTT AAC AGG AGA ACT AAC AAG GCC GAG CTC AAG AGA GTA	2909
	Val Pro Ser Phe Asn Arg Arg Thr Asn Lys Ala Glu Leu Lys Arg Val	
	905 910 915	
10	AAG GCC CTG ATT AGG CAC GAT TAT TCT GCC AAG GTC TAC ACC ATC AGA	2957
	Lys Ala Leu Ile Arg His Asp Tyr Ser Gly Lys Val Tyr Thr Ile Arg	
	920 925 930	
15	CTG AAG TCG GGG AGG AGA ATA AAG ATA ACC TCT GGC CAC AGC CTC TTC	3005
	Leu Lys Ser Gly Arg Arg Ile Lys Ile Thr Ser Gly His Ser Leu Phe	
	935 940 945 950	
20	TCT GTG AGA AAC GGG GAG CTC GTT GAA GTT ACG GGC GAT GAA CTA AAG	3053
	Ser Val Arg Asn Gly Glu Leu Val Glu Val Thr Gly Asp Glu Leu Lys	
	955 960 965	
25	CCA GGT GAC CTC GTT GCA GTC CCG CGG AGA TTG GAG CTT CCT GAG AGA	3101
	Pro Gly Asp Leu Val Ala Val Pro Arg Arg Leu Glu Leu Pro Glu Arg	
	970 975 980	
30	AAC CAC GTG CTG AAC CTC GTT GAA CTG CTC CTT GGA ACG CCA GAA GAA	3149
	Asn His Val Leu Asn Leu Val Glu Leu Leu Leu Gly Thr Pro Glu Glu	
	985 990 995	
35	GAA ACT TTG GAC ATC GTC ATG ACG ATC CCA GTC AAG GGT AAG AAG AAC	3197
	Glu Thr Leu Asp Ile Val Met Thr Ile Pro Val Lys Gly Lys Lys Asn	
	1000 1005 1010	
40	TTC TTT AAA GGG ATG CTC AGG ACT TTG CGC TGG ATT TTC GGA GAG GAA	3245
	Phe Phe Lys Gly Met Leu Arg Thr Leu Arg Trp Ile Phe Gly Glu Glu	
	1015 1020 1025 1030	
45	AAG AGG CCC AGA ACC GCG AGA CGC TAT CTC AGG CAC CTT GAG GAT CTC	3293
	Lys Arg Pro Arg Thr Ala Arg Arg Tyr Leu Arg His Leu Glu Asp Leu	
	1035 1040 1045	
50		
55		

	GGC TAT GTC CGG CTT AAG AAG ATC GGC TAC GAA GTC CTC GAC TGG GAC	3341
5	Gly Tyr Val Arg Leu Lys Lys Ile Gly Tyr Glu Val Leu Asp Trp Asp	
	1050 1055 1060	
	TCA CTT AAG AAC TAC AGA AGG CTC TAC GAG GCG CTT GTC GAG AAC GTC	3389
10	Ser Leu Lys Asn Tyr Arg Arg Leu Tyr Glu Ala Leu Val Glu Asn Val	
	1065 1070 1075	
	AGA TAC AAC GGC AAC AAG AGG GAG TAC CTC GTT GAA TTC AAT TCC ATC	3437
15	Arg Tyr Asn Gly Asn Lys Arg Glu Tyr Leu Val Glu Phe Asn Ser Ile	
	1080 1085 1090	
	CGG GAT GCA GTT GGC ATA ATG CCC CTA AAA GAG CTG AAG GAG TGG AAG	3485
20	Arg Asp Ala Val Gly Ile Met Pro Leu Lys Glu Leu Lys Glu Trp Lys	
	1095 1100 1105 1110	
	ATC GGC ACG CTG AAC GGC TTC AGA ATG AGA AAG CTC ATT GAA GTG GAC	3533
25	Ile Gly Thr Leu Asn Gly Phe Arg Met Arg Lys Leu Ile Glu Val Asp	
	1115 1120 1125	
	GAG TCG TTA GCA AAG CTC CTC GGC TAC TAC GTG AGC GAG GGC TAT GCA	3581
30	Glu Ser Leu Ala Lys Leu Leu Gly Tyr Tyr Val Ser Glu Gly Tyr Ala	
	1130 1135 1140	
	AGA AAG CAG AGG AAT CCC AAA AAC GGC TGG AGC TAC AGC GTG AAG CTC	3629
35	Arg Lys Gln Arg Asn Pro Lys Asn Gly Trp Ser Tyr Ser Val Lys Leu	
	1145 1150 1155	
	TAC AAC GAA GAC CCT GAA GTG CTG GAC GAT ATG GAG AGA CTC GCC AGC	3677
40	Tyr Asn Glu Asp Pro Glu Val Leu Asp Asp Met Glu Arg Leu Ala Ser	
	1160 1165 1170	
	AGG TTT TTC GGG AAG GTG AGC CGG GGC AGG AAC TAC GTT GAG ATA CCG	3725
45	Arg Phe Phe Gly Lys Val Arg Arg Gly Arg Asn Tyr Val Glu Ile Pro	
	1175 1180 1185 1190	
	AAG AAG ATC GGC TAC CTC TTT GAG AAC ATG TGC GGT GTC CTA GCG	3773
50	Lys Lys Ile Gly Tyr Leu Leu Phe Glu Asn Met Cys Gly Val Leu Ala	
55		

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	1195	1200	1205	
	GAG AAC AAG AGG ATT CCC GAG TTC GTC TTC ACG TCC CCG AAA GGG GTT			3821
5	Glu Asn Lys Arg Ile Pro Glu Phe Val Phe Thr Ser Pro Lys Gly Val			
	1210	1215	1220	
	CGG CTG GCC TTC CTT GAG GGG TAC TCA TCG GCG ATG GCG ACG TCC ACC			3869
10	Arg Leu Ala Phe Leu Glu Gly Tyr Ser Ser Ala Met Ala Thr Ser Thr			
	1225	1230	1235	
	GAA CAA GAG ACT CAG GCT CTC AAC GAA AAG CGA GCT TTA GCG AAC CAG			3917
15	Glu Gln Glu Thr Gln Ala Leu Asn Glu Lys Arg Ala Leu Ala Asn Gln			
	1240	1245	1250	
	CTC GTC CTC CTC TTG AAC TCG GTG GGG GTC TCT GCT GTA AAA CTT GGG			3965
20	Leu Val Leu Leu Leu Asn Ser Val Gly Val Ser Ala Val Lys Leu Gly			
	1255	1260	1265	1270
	CAC GAC AGC GGC GTT TAC AGG GTC TAT ATA AAC GAG GAG CTC CCG TTC			4013
25	His Asp Ser Gly Val Tyr Arg Val Tyr Ile Asn Glu Glu Leu Pro Phe			
	1275	1280	1285	
	GTA AAG CTG GAC AAG AAA AAG AAC GCC TAC TAC TCA CAC GTG ATC CCC			4061
30	Val Lys Leu Asp Lys Lys Lys Asn Ala Tyr Tyr Ser His Val Ile Pro			
	1290	1295	1300	
	AAG GAA GTC CTG AGC GAG GTC TTT GGG AAG GTT TTC CAG AAA AAC GTC			4109
35	Lys Glu Val Leu Ser Glu Val Phe Gly Lys Val Phe Gln Lys Asn Val			
	1305	1310	1315	
	AGT CCT CAG ACC TTC AGG AAG ATG GTC GAG GAC GGA AGA CTC GAT CCC			4157
40	Ser Pro Gln Thr Phe Arg Lys Met Val Glu Asp Gly Arg Leu Asp Pro			
	1320	1325	1330	
	GAA AAG GCC CAG AGG CTC TCC TGG CTC ATT GAG GGG GAC GTA GTG CTC			4205
	Glu Lys Ala Gln Arg Leu Ser Trp Leu Ile Glu Gly Asp Val Val Leu			
	1335	1340	1345	1350
	GAC CGC GTT GAG TCC GTT GAT GTG GAA GAC TAC GAT GGT TAT GTC TAT			4253
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	TGG AGC GAG ATA GCG AAA GAG ACG CAG GCG AGG GTT CTT GAA GCT TTG	4733
5	Trp Ser Glu Ile Ala Lys Glu Thr Gln Ala Arg Val Leu Glu Ala Leu	
	1515 1520 1525	
	CTA AAG GAC GGT GAC GTC GAG AAG GCC GTG AGG ATA GTC AAA GAA GTT	4781
10	Leu Lys Asp Gly Asp Val Glu Lys Ala Val Arg Ile Val Lys Glu Val	
	1530 1535 1540	
	ACC GAA AAG CTG AGC AAG TAC GAG GTT CCG CCG GAG AAG CTG GTG ATC	4829
15	Thr Glu Lys Leu Ser Lys Tyr Glu Val Pro Pro Glu Lys Leu Val Ile	
	1545 1550 1555	
	CAC GAG CAG ATA ACG AGG GAT TTA AAG GAC TAC AAG GCA ACC GGT CCC	4877
20	His Glu Gln Ile Thr Arg Asp Leu Lys Asp Tyr Lys Ala Thr Gly Pro	
	1560 1565 1570	
	CAC GTT GCC GTT GCC AAG AGG TTG GCC GCG AGA GGA GTC AAA ATA CGC	4925
25	His Val Ala Val Ala Lys Arg Leu Ala Ala Arg Gly Val Lys Ile Arg	
	1575 1580 1585 1590	
	CCT GGA ACG GTG ATA AGC TAC ATC GTG CTC AAG GGC TCT GGG AGG ATA	4973
30	Pro Gly Thr Val Ile Ser Tyr Ile Val Leu Lys Gly Ser Gly Arg Ile	
	1595 1600 1605	
	GGC GAC AGG GCG ATA CCG TTC GAC GAG TTC GAC CCG ACG AAG CAC AAG	5021
35	Gly Asp Arg Ala Ile Pro Phe Asp Glu Phe Asp Pro Thr Lys His Lys	
	1610 1615 1620	
	TAC GAC GCC GAG TAC TAC ATT GAG AAC CAG GTT CTC CCA GCC GTT GAG	5069
40	Tyr Asp Ala Glu Tyr Tyr Ile Glu Asn Gln Val Leu Pro Ala Val Glu	
	1625 1630 1635	
	AGA ATT CTG AGA GCC TTC GGT TAC CGC AAG GAA GAC CTG CGC TAC CAG	5117
45	Arg Ile Leu Arg Ala Phe Gly Tyr Arg Lys Glu Asp Leu Arg Tyr Gln	
	1640 1645 1650	
	AAG ACG AGA CAG GTT GGT TTG AGT GCT TGG CTG AAG CCG AAG GGA ACT	5165
50	Lys Thr Arg Gln Val Gly Leu Ser Ala Trp Leu Lys Pro Lys Gly Thr	

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1655 1660 1665 1670
 5 TGACCTTTCC ATTTGTTTTC CAGCGGATAA CCCTTTAACT TCCCTTTCAA AAACCTCCCT 5225
 TAGGGAAAGA CCATGAAGAT AGAAATCCGG CGGCGCCCGG TTAAATACGC TAGGATAGA 5285
 GTGAAGCCAG ACGGCAGGGT AGTCGTCACT GCCCCGAGGG TTCAACGTTG AGAAGTT 5342

10 SEQ ID No.6

Length: 5342 base pairs

Type: nucleic acid (DNA)

15 Strandedness: double

Topology: linear

Molecular Type: cDNA

20 Source: hyperthermophilic archaeon

Strain name: KOD1

25 Sequence Description:

30 GCTTGAGGGC CTGCGGTTAT GGGACGTTGC AGTTTGGGCC TACTCAAAGA TGCCGGTTTT 60
 ATAACGGAGA AAAATGGGGA GCTATTACGA TCTCTCCTTG ATGTGGGGTT TACAATAAAG 120
 CCTGGATTGT TCTACAAGAT TATGGGGGAT GAAAGATGAT CCTCGACACT GACTACATAA 180
 CCGAGGATGG AAAGCCTGTC ATAAGAATTT TCAAGAAGGA AAACGGCGAG TTAAAGATTG 240
 35 AGTACGACCG GACTTTTGAA CCCTACTTCT ACGCCCTCCT GAAGGACGAT TCTGCCATTG 300
 AGGAAGTCAA GAAGATAACC GCCGAGAGGC ACGGACGGT TGTAACGTTT AAGCGGGTTG 360
 AAAAGGTTCA GAAGAAGTTC CTCGGGAGAC CAGTTGAGGT CTGGAAACTC TACTTTACTC 420
 40 ATCCGCAGGA CGTCCCAGCG ATAAGGGACA AGATACGAGA GCATGGAGCA GTTATTGACA 480
 TCTACGAGTA CGACATACCC TTCGCCAAGC GCTACCTCAT AGACAAGGGA TTAGTGCCAA 540
 TGGAAGCGCA CGAGGAGCTG AAAATGCTCG CCTTCGACAT TCAAACCTCTC TACCATGAGG 600
 45 GCGAGGAGTT CCGCGAGGGG CCAATCCTTA TGATAAGCTA CGCCGACCAG GAAGGGGCCA 660
 GGGTGATAAC TTGGAAGAAC GTGGATCTCC CCTACGTTGA CGTCGTCTCG ACGGAGAGGG 720
 AGATGATAAA GCGCTTCCTC CGTGTGTGA AGGAGAAAGA CCCGGACGTT CTCATAACCT 780
 50 ACAACGGCGA CAACTTCGAC TTCGCCTATC TGAAAAAGCG CTGTGAAAAG CTCGGAATAA 840
 ACTTCGCCCT CGGAAGGGAT GGAAGCGAGC CGAAGATTCA GAGGATGGGC GACAGGTTTG 900

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CCGTCGAAGT GAAGGGACGG ATACACTTCG ATCTCTATCC TGTGATAAGA CGGACGATAA 960
ACCTGCCCCAC ATACACGCTT GAGGCCGTTT ATGAAGCCGT CTTGGGTCAG CCGAAGGAGA 1020
5 AGGTTTACGC TGAGGAAATA ACACCAGCCT GGGAAACCGG CGAGAACCCTT GAGAGACTCG 1080
CCCCTACTC GATGGAAGAT GCGAAGGTCA CATACGAGCT TGGGAAGGAG TTCCTTCCGA 1140
10 TGGAGGCCCA GCTTTCTCGC TTAATCGGCC AGTCCCTCTG GGACGTCTCC CGCTCCAGCA 1200
CTGGCAACCT CGTTGAGTGG TTCCTCCTCA GGAAGGCCCT ATGAGAGGAA TGAGCTGGCC 1260
CCGAACAAGC CCGATGAAAA GGAGCTGGCC AGAAGACGGC AGAGCTATGA AGGAGGCTAT 1320
15 GTAAAAGAGC CCGAGAGAGG GTTGTGGGAG AACATAGTGT ACCTAGATTT TAGATGCCAT 1380
CCAGCCGATA CGAAGGTTGT CGTCAAGGGG AAGGGGATTA TAAACATCAG CGAGGTTTCA 1440
GAAGGTGACT ATGTCTTGG GATTGACGGC TGGCAGAGAG TTAGAAAAGT ATGGGAATAC 1500
20 GACTACAAAG GGGAGCTTGT AAACATAAAC GGGTTAAAGT GTACGCCCAA TCATAAGCTT 1560
CCCGTTGTTA CAAAGAACGA ACGACAAACG AGAATAAGAG ACAGTCTTGC TAAGTCTTTC 1620
CTTACTAAAA AAGTTAAGG CAAGATAATA ACCACTCCCC TTTTCTATGA AATAGGCAGA, 1680
25 GCGACAAGTG AGAATATTCC AGAAGAAGAG GTTCTCAAGG GAGAGCTCGC TGGCATAGTA, 1740
TTGGCTGAAG GAACGCTCTT GAGGAAAGAC GTTGAATACT TTGATTCATC CCGCAAAAAA 1800
CGGAGGATTT CACACCAGTA TCGTGTGAG ATAACCATTG GGAAAGACGA GGAGGAGTTT 1860
30 AGGGATCGTA TCACATACAT TTTTGAGCGT TTGTTTGGGA TTA CTCCAAG CATCTCGGAG 1920
AAGAAAGGAA CTAACGCAGT AACACTCAAA GTTGCGAAGA AGAATGTTTA TCTTAAAGTC 1980
35 AAGGAAATTA TGGACAACAT AGAGTCCCTA CATGCCCCCT CGGTTCTCAG GGGATTCTTC 2040
GAAGGCGACC GTTCAGTAAA CAGGTTAGGA GGAGTATTGT TGCAACCCAG GGTACAAAGA 2100
ACGAGTGGAA GATTAAACTG GTGTCAAAAC TGCTCTCCCA GCTTGGTATC CCTCATCAAA 2160
40 CGTACACGTA TCAGTATCAG GAAAATGGGA AAGATCGGAG CAGGTATATA CTGGAGATAA 2220
CTGGAAGGA CGGATTGATA CTGTTCCAAA CACTCATTGG ATTCAATCAGT GAAAGAAAGA 2280
ACGCTCTGCT TAATAAGGCA ATATCTCAGA GGGAAATGAA CAACTTGGA AACAATGGAT 2340
45 TTTACAGGCT CAGTGAATTC AATGTCAGCA CGGAATACTA TGAGGGCAAG GTCTATGACT 2400
TAACTCTTGA AGGAACTCCC TACTTTGCCA ATGGCATATT GACCCATAAC TCCCTGTACC 2460
CCTCAATCAT CATCACCCAC AACGTCTCGC CGGATACGCT CAACAGAGAA GGATGCAAGG 2520
50 AATATGACGT TGCCCCACAG GTCGGCCACC GCTTCTGCAA GGA CTCCA GGATTTATCC 2580
CGAGCCTGCT TGGAGACCTC CTAGAGGAGA GGCAGAAGAT AAAGAAGAAG ATGAAGGCCA 2640

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CGATTGACCC GATCGAGAGG AAGCTCCTCG ATTACAGGCA GAGGGCCATC AAGATCCTGG 2700
 CAAACAGCAT CCTACCCGAG GAATGGCTTC CAGTCTCCGA GGAAGGGGAG GTTCACTTCG 2760
 TCAGGATTGG AGAGCTCATA GACCGGATGA TGGAGGAAAA TGCTGGGAAA GTAAAGAGAG 2820
 AGGGCGAGAC GGAAGTGCTT GAGGTCAGTG GGCTTGAAGT CCCGTCTTTT AACAGGAGAA 2880
 CTAACAAGGC CGAGCTCAAG AGAGTAAAGG CCCTGATTAG GCACGATTAT TCTGGCAAGG 2940
 TCTACCCAT CAGACTGAAG TCGGGGAGGA GAATAAGAT AACCTCTGGC CACAGCCTCT 3000
 TCTCTGTGAG AAACGGGGAG CTCGTTGAAG TTACGGGCGA TGAATAAAG CCAGGTGACC 3060
 TCGTTGCAGT CCCGCGGAGA TTGGAGCTTC CTGAGAGAAA CCACGTGCTG AACCTCGTTG 3120
 AACTGCTCCT TGGAAACGCCA GAAGAAGAAA CTTTGGACAT CGTCATGACG ATCCCAGTCA 3180
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 CGAAGAAGAT CGGCTACCTG CTCTTTGAGA ACATGTGCGG TGTCCTAGCG GAGAACAAGA 3780
 GGATTCCCGA GTTCGTCTTC ACGTCCCCGA AAGGGGTTCG, GCTGGCCTTC CTTGAGGGGT 3840
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 CTTTAGCGAA CCAGCTCGTC CTCCTCTTGA ACTCGGTGGG GGTCTCTGCT GTAAACTTG 3960
 GGCACGACAG CGGCGTTTAC AGGCTCTATA TAAACGAGGA GCTCCCGTTC GTAAAGCTGG 4020
 ACAAGAAAAA GAACGCCTAC TACTCACACG TGATCCCCAA GGAAGTCCTG AGCGAGGTCT 4080
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 GAAGACTCGA TCCCGAAAAG GCCCAGAGGC TCTCCTGGCT CATTGAGGGG GACGTAGTGC 4200
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 TCGAGGACAA CGAGAACTTC CTCGTTGGCT TTGGGTTGGT CTATGCTCAC AACAGCTACT 4320
 ACGGTTACTA CGGCTATGCA AGGGCGCGCT GGTACTGCAA GGAGTGTGCA GAGAGCGTAA 4380

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CGGCCTGGGG AAGGGAGTAC ATAACGATGA CCATCAAGGA GATAGAGGAA AAGTACGGCT 4440
 5 TTAAGGTAAT CTACAGCGAC ACCGACGGAT TTTTGGCCAC AATACCTGGA GCCGATGCTG 4500
 AAACCGTCAA AAAGAAGGCT ATGGAGTTCC TCAACTATAT CAACGCCAAA CTTCCGGGCG 4560
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 10 ATGCGGTGAT AGACGAGGAA GGCAAGATAA CAACGCGCGG ACTTGAGATT GTGAGGCGTG 4680
 ACTGGAGCGA GATAGCGAAA GAGACGCAGG CGAGGGTTCT TGAAGCTTTG CTAAAGCACC 4740
 15 GTGACGTCGA GAAGGCCGTG AGGATAGTCA AAGAAGTTAC CGAAAAGCTG AGCAAGTACC 4800
 AGGTTCCGCC GGAGAAGCTG GTGATCCACG AGCAGATAAC GAGGGATTTA AAGGACTACA 4860
 AGGCAACCGG TCCCCACGTT GCCGTTGCCA AGAGGTTGGC CGCGAGAGGA GTCAAAATAC 4920
 20 GCCCTGGAAC GGTGATAAGC TACATCGTGC TCAAGGGCTC TGGGAGGATA GCGACAGGG 4980
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 25 AGAACCAGGT TCTCCCAGCC GTTGAGAGAA TTCTGAGAGC CTTCCGTTAC CGCAAGGAAG 5100
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 30 CTTGACCTTT CCATTTGTTT TCCAGCGGAT AACCCTTTAA CTTCCCTTTC AAAAATCCC 5220
 TTTAGGAAA GACCATGAAG ATAGAAATCC GCGGCGCCCC GGTAAATAC GCTAGGATAG 5280
 AAGTGAAGCC AGACGGCAGG GTAGTCGTCA CTGCCCCGAG GGTCAACGT TGAGAAGTT 5339

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SEQ ID No.7

Length: 24 base pairs

Type: nucleic acid

Topology: linear

Molecular Type: synthetic DNA

Sequence Description:

GGATTAGTGC CAATGGAAGG CGAC

24

SEQ ID No.8

Length: 24 base pairs

Type: nucleic acid

Topology: linear

Molecular Type: synthetic DNA

Sequence Description:

GAGGGCGAAG TTTATTCCGA GCTT

24

SEQ ID No.9

Length: 324 base pairs

Type: nucleic acid (DNA)

Strandedness: double

Topology: linear

Molecular Type: cDNA

Sequence Description:

GGATTAGTGC CAATGGAAGG CGACGAGGAG CTGAAAATGC TCGCCTTCGA CATTCAAACCT 60

CTCTACCATG AGGGCGAGGA GTTCGCCGAG GGGCCAATCC TTATGATAAG CTACGCCGAC 120

GAGGAAGGGG CCAGGGTGAT AACTTGGAAG AACGTGGATC TCCCCTACGT TGACGTCGTC 180

TCGACGGAGA GGGAGATGAT AAAGCGCTTC CTCCGTGTTG TGAAGGAGAA AGACCCGGAC 240

GTTCTCATAA CCTACAACGG CGACAACCTC GACTTCGCCT ATCTGAAAAA GCGCTGTGAA 300

AAGCTCGGAA TAAACTTCGC CCTC 324

SEQ ID No.10

Length: 108 base pairs

Type: amino acid

Topology: linear

Molecular Type: protein

Sequence Description:

Gly Leu Val Pro Met Glu Gly Asp Glu Glu Leu Lys Met Leu Ala Phe

1 5 10 15

Asp Ile Gln Thr Leu Tyr His Glu Gly Glu Glu Phe Ala Glu Gly Pro

20 25 30

Ile Leu Met Ile Ser Tyr Ala Asp Glu Glu Gly Ala Arg Val Ile Thr

35 40 45

Trp Lys Asn Val Asp Leu Pro Tyr Val Asp Val Val Ser Thr Glu Arg

50 55 60

Glu Met Ile Lys Arg Phe Leu Arg Val Val Lys Glu Lys Asp Pro Asp

65 70 75 80

Val Leu Ile Thr Tyr Asn Gly Asp Asn Phe Asp Phe Ala Tyr Leu Lys

85 90 95

Lys Arg Cys Glu Lys Leu Gly Ile Asn Phe Ala Leu

100 105

SEQ ID No.11

Length: 42 base pairs

Type: nucleic acid (DNA)

Strandedness: single

Molecular Type: synthetic DNA

Sequence Description:

GCCATCAAGA TCCTGGCAAA CAGCTACTAC GGTTACTACG GC 42

SEQ ID No.12

Length: 32 base pairs

Type: nucleic acid (DNA)

Strandedness: single

Molecular Type: synthetic DNA

Sequence Description:

GATGGATCCA ACTTCTCAAC GTTGAACCCT CG 32

SEQ ID No.13

Length: 46 base pairs

Type: nucleic acid (DNA)

Strandedness: single

Molecular Type: synthetic DNA

Sequence Description:

GAACATAGTG TACCTAGATT TTAGATCCCT GTACCCCTCA ATCATC 46

SEQ ID No.14

Length: 42 base pairs

Type: nucleic acid (DNA)

Strandedness: single

Molecular Type: synthetic DNA

Sequence Description:

CCCGTAGTAA CCGTAGTAGC TGTTTGCCAG GATCTTGATG GC 42

SEQ ID No.15

Length: 33 base pairs

Type: nucleic acid (DNA)

Strandedness: single

Molecular Type: synthetic DNA

Sequence Description:

ATCGATATCC TCGACACTGA CTACATAACC GAG

33

SEQ ID No.16

Length: 46 base pairs

Type: nucleic acid (DNA)

Strandedness: single

Molecular Type: synthetic DNA

Sequence Description:

GATGATTGAG GGGTACAGGG ATCTAAAATC TAGGTACACT ATGTTC

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Claims

1. A DNA comprising a nucleotide sequence that encodes a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity derived from a hyperthermophilic archaeon strain.
2. The DNA of claim 1, wherein the strain is KOD1.
3. The DNA of claim 1 or 2, wherein a nucleotide sequence encodes the thermostable DNA polymerase which has the amino acid sequence of SEQ ID No. 1.
4. The DNA of any one of claims 1 to 3, wherein said DNA has a nucleotide sequence of SEQ ID No. 5.
5. A DNA encoding a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity, said DNA comprising a nucleotide sequence:
 - (a) which differs from a DNA of claim 4 in codon sequence due to the degeneracy of the genetic code;
 - (b) which hybridizes with a DNA of claim 4 or section (a), above; or
 - (c) represents a fragment, allelic or other variation of a DNA of claim 4.
6. A recombinant DNA expression vector that comprises the DNA sequence of any one of claims 1 to 5.
7. The recombinant DNA expression vector of claim 6, in which the vector is a vector derived from pET-8c.
8. A recombinant host cell which is transformed by a recombinant DNA expression vector of claim 7.
9. The recombinant host cell of claim 8, in which the host cell is Escherichia coli.

10. A thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity which is encoded by the DNA of any one of claims 1 to 5.
11. The thermostable DNA polymerase of claim 10, which has the following physical and chemical properties:
Action: It catalyses the extension reaction of nucleotide sequence that is complementary to a template nucleotide sequence using nucleotide triphosphates as substrate and it has a 3'-5' exonuclease activity,
DNA extension rate: at least 30 bases/second
Optimum pH: 6.5 - 7.5 (at 75°C)
Optimum temperature: 75°C
Molecular weight: about 88 - 90 kDa
Amino acid sequence: as defined in SEQ ID No. 1.
12. A method for producing a DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity comprising culturing the recombinant host cell of claim 8 or 9.
13. A method for purifying a DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity comprising culturing the recombinant host cell of claim 8 or 9, and further (a) recovering the cultured recombinant host cells, disintegrating them and preparing the cell extract, and (b) removing the impurified proteins derived from recombinant host cells.
14. The DNA polymerase of claim 10 or 11, which is obtainable by using the recombinant host cells of claim 8 or 9 or by the method of claims 12 or 13.
15. A method for amplifying a target nucleic acid comprising reacting the target nucleic acid with four kinds of dNTP and primer sufficiently complementary to said target nucleic acid in a buffer solution which contains a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity such that the above mentioned primer is annealed to the target nucleic acid and an extension product is synthesized from the primer.
16. A method for amplifying a target nucleic acid in a sample wherein each target nucleic acid consists of two separate complementary strands which comprises the following steps (a) to (d), characterized in that a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity is used as a thermostable DNA polymerase;
(a) modifying the target nucleic acid, if necessary, to produce single-stranded nucleic acids;
(b) reacting the single-stranded nucleic acids with four kinds of dNTP and primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, in a buffer solution which contains said thermostable DNA polymerase such that the above mentioned primers are annealed to the single-stranded nucleic acids and extension products are synthesized from the primers;
(c) separating the primer extension products from the templates on which they are synthesized to produce single-stranded nucleic acids; and
(d) repeatedly conducting the above mentioned steps (b) and (c).
17. A method for detecting a target nucleic acid in a sample wherein each target nucleic acid consists of two separate complementary strands which comprises the following steps (a) to (e), characterized in that a thermostable DNA polymerase having a DNA extension rate of at least 30 bases/second and a 3'-5' exonuclease activity is used as a thermostable DNA polymerase:
(a) modifying the target nucleic acid, if necessary, to produce single-stranded nucleic acids;
(b) reacting the single-stranded nucleic acids with four kinds of dNTP and primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, in a buffer solution which contains said thermostable DNA polymerase such that the above mentioned primers are annealed to the single-stranded nucleic acids and extension products are synthesized from the primers;
(c) separating the primer extension products from the templates on which they are synthesized to produce single-stranded nucleic acids;
(d) repeatedly conducting the above mentioned steps (b) and (c); and
(e) detecting an amplified nucleic acid.
18. The method of any one of claims 15 to 17, wherein said thermostable DNA polymerase is encoded by the DNA of

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any one of claims 1 to 5 or produced by the method of claim 12 or 13.

5 19. A reagent kit for amplifying target nucleic acid which comprises primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, four kinds of dNTP, divalent cation and the thermostable DNA polymerase encoded by the DNA of any one of claims 1 to 5 or produced by the method of claim 12 or 13.

10 20. A reagent kit for detecting target nucleic acid which comprises primers, wherein said primers are selected so as to be sufficiently complementary to different strands of target nucleic acid to anneal therewith, four kinds of dNTP, divalent cation, thermostable DNA polymerase encoded by the DNA of any one of claims 1 to 5 or produced by the method of claim 12 or 13, amplifying buffer solution, a probe capable of hybridizing with amplified nucleic acid and a detection buffer solution.

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Fig. 1

Measurement of the DNA Extension Rate of DNA Polymerase
Derived from the Hyperthermophilic Archaeon Strain KOD1

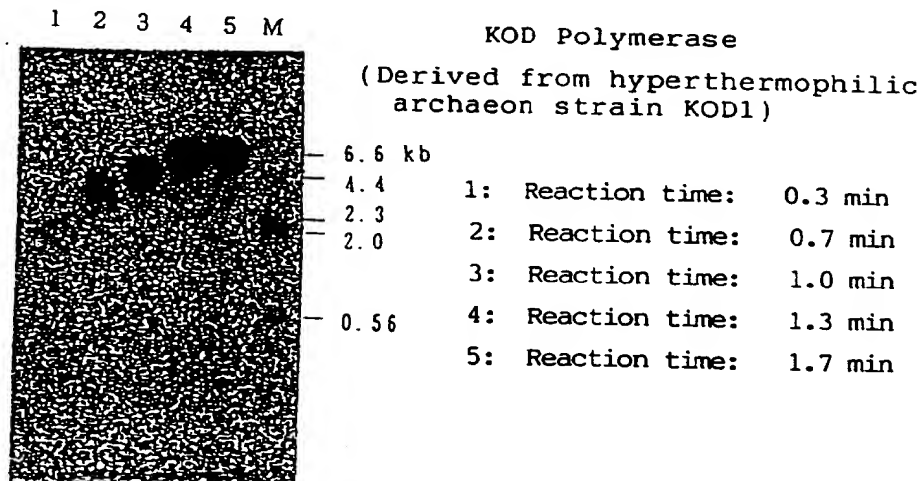
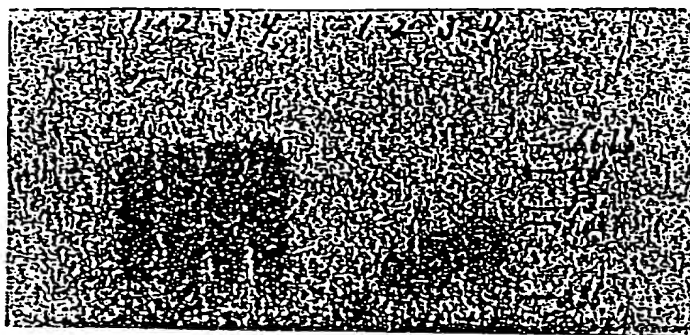


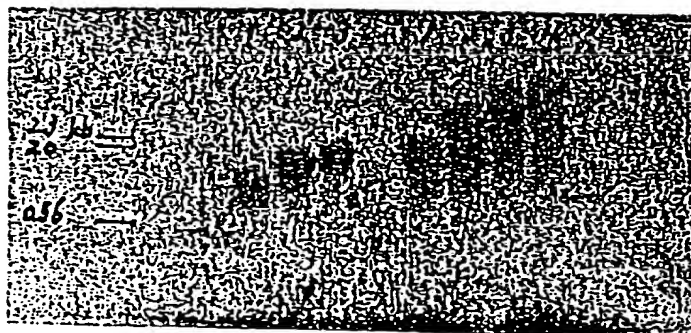
Fig. 2

Comparison of DNA Extension Rates of Various Thermostable
DNA Polymerases



2 a

KOD Polymerase		Pfu Polymerase	
1:	Reaction Time: 0.7 min	1:	Reaction Time: 1.0 min
2:	" : 1.0 min	2:	" : 1.5 min
3:	" : 1.3 min	3:	" : 2.0 min
4:	" : 1.7 min	4:	" : 2.5 min

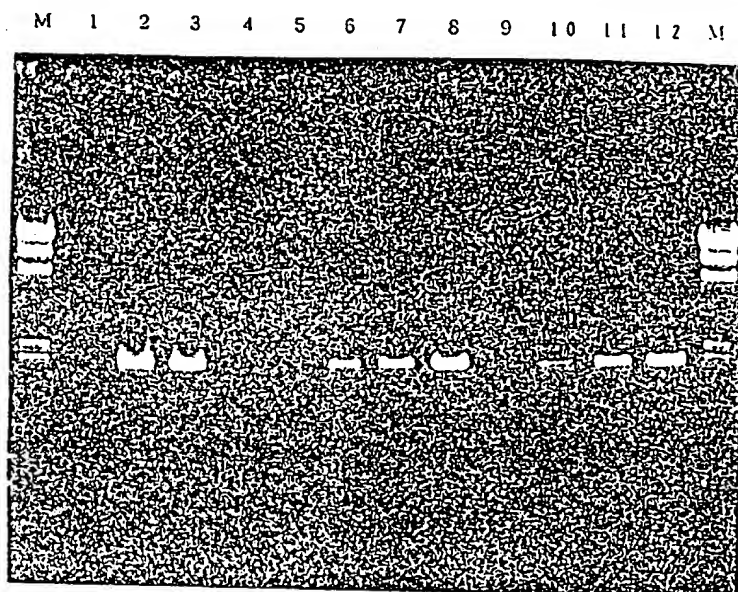


2 b

Deep Vent Polymerase		Taq Polymerase	
1:	Reaction Time: 1.5 min	1:	Reaction Time: 1.0 min
2:	" : 2.0 min	2:	" : 1.5 min
3:	" : 2.5 min	3:	" : 2.0 min
		4:	" : 2.5 min

Fig. 3

Comparison of the PCR due to the Difference in the Reaction Time
of Various Thermostable DNA Polymerases



- | | | |
|-----|--|-----------------------|
| 1: | KOD Polymerase (Derived from Hyperthermophilic archaeon strain KOD1) | Reaction Time: 1 sec. |
| 2: | KOD Polymerase (Derived from Hyperthermophilic archaeon strain KOD1) | " " : 5 sec. |
| 3: | KOD Polymerase (Derived from Hyperthermophilic archaeon strain KOD1) | " " : 10 sec |
| 4: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | Reaction Time: 1 sec. |
| 5: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 5 sec. |
| 6: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 10 sec. |
| 7: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 30 sec. |
| 8: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 60 sec. |
| 9: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 30 sec. |
| 10: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 60 sec. |
| 11: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 90 sec. |
| 12: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 120 sec. |

Fig. 4

Construction of Expression Recombinant Vector (pET-pol)

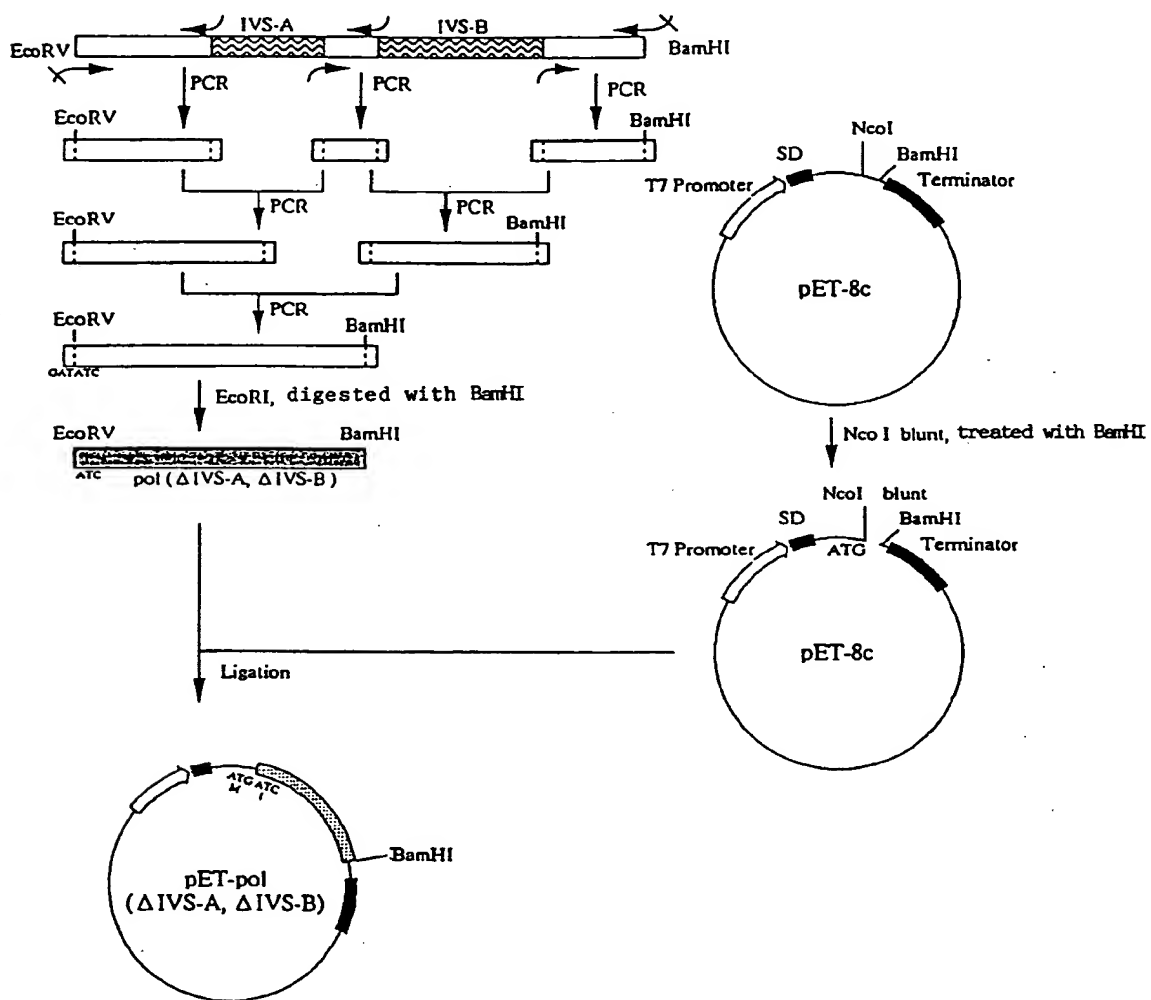
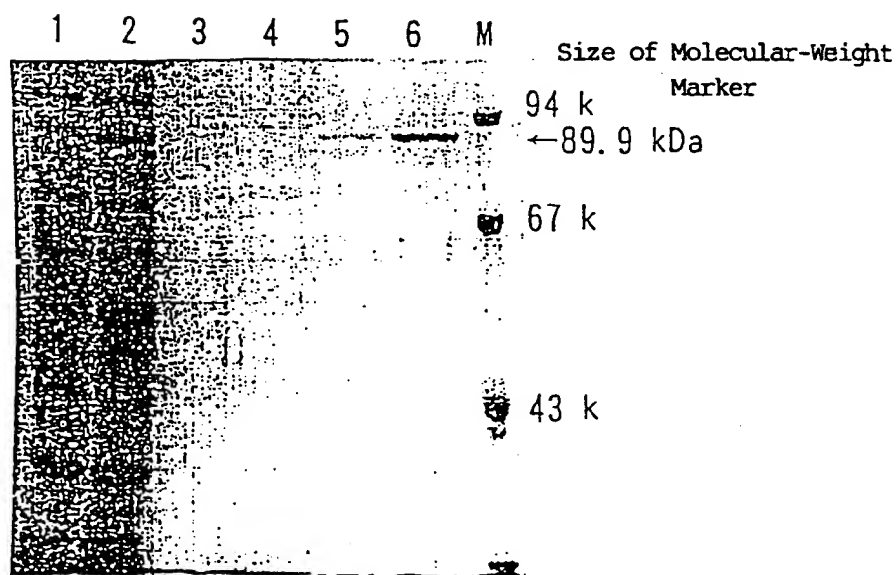
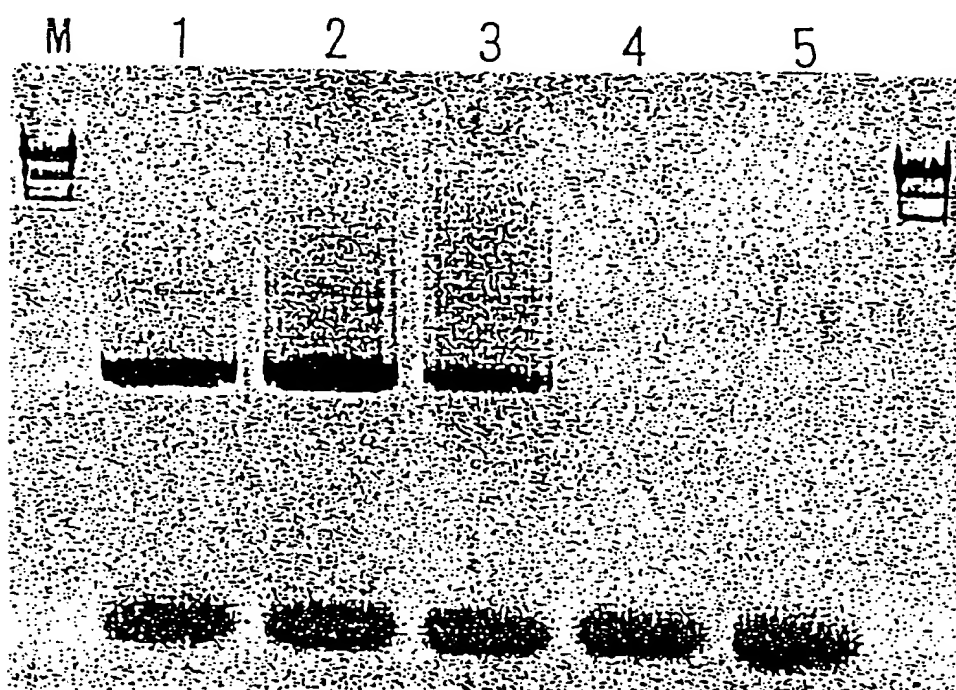


Fig. 5



- 1: pET-8c Precipitate
- 2: pET-pol (Δ IVS-A, Δ IVS-B) Precipitate
- 3: pET-8c Supernatant Liquid
- 4: pET-8c Supernatant Liquid x 5
- 5: pET-pol (Δ IVS-A, Δ IVS-B) Supernatant Liquid
- 6: pET-pol (Δ IVS-A, Δ IVS-B) Supernatant Liquid x 5

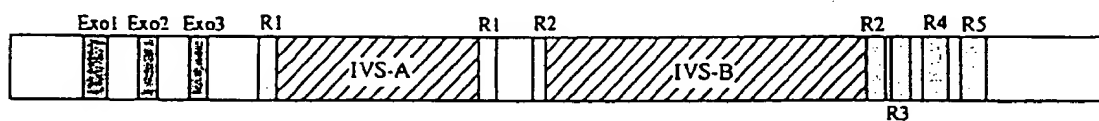
Fig. 6



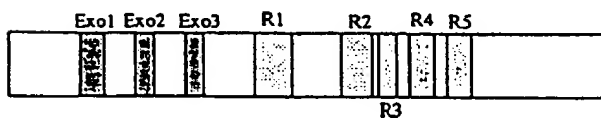
- 1: Vent Polymerase (Derived from *Thermococcus litoralis*)
- 2: pET-pol (Δ IVS-A, Δ IVS-B) Supernatant Liquid
- 3: pET-pol (Δ IVS-A, Δ IVS-B) Supernatant Liquid x 5
- 4: pET-8c Supernatant Liquid
- 5: pET-8c Supernatnat Liquid x 5

Fig. 7

DNA Polymerase Gene of Hyperthermophilic Archaeon Strain KOD1



DNA Polymerase Gene of *Pyrococcus furiosus* (Pfu DNA Polymerase)



DNA Polymerase Gene of *Thermococcus litoralis* (Vent DNA Polymerase)

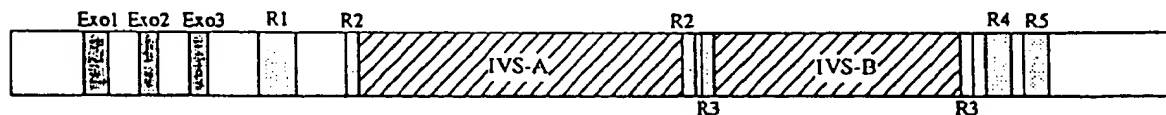


Fig. 1

Measurement of the DNA Extension Rate of DNA Polymerase
Derived from the Hyperthermophilic Archaeon Strain KOD1

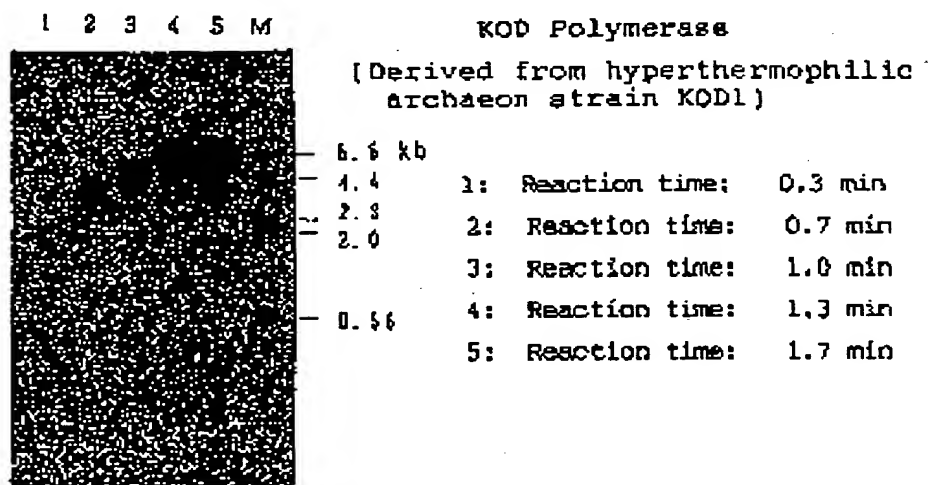
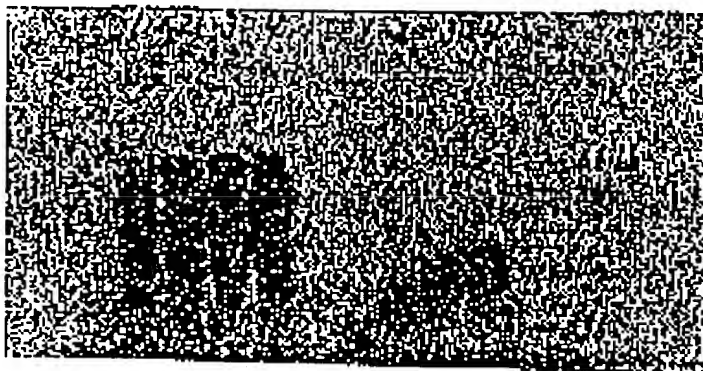


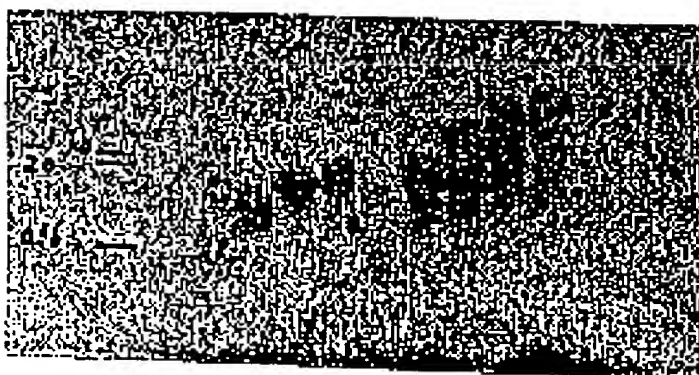
Fig. 2

Comparison of DNA Extension Rates of Various Thermostable
DNA Polymerases



2 a

KOD Polymerase		Pfu Polymerase	
1:	Reaction Time: 0.7 min	1:	Reaction Time: 1.0 min
2:	" : 1.0 min	2:	" : 1.5 min
3:	" : 1.3 min	3:	" : 2.0 min
4:	" : 1.7 min	4:	" : 2.5 min

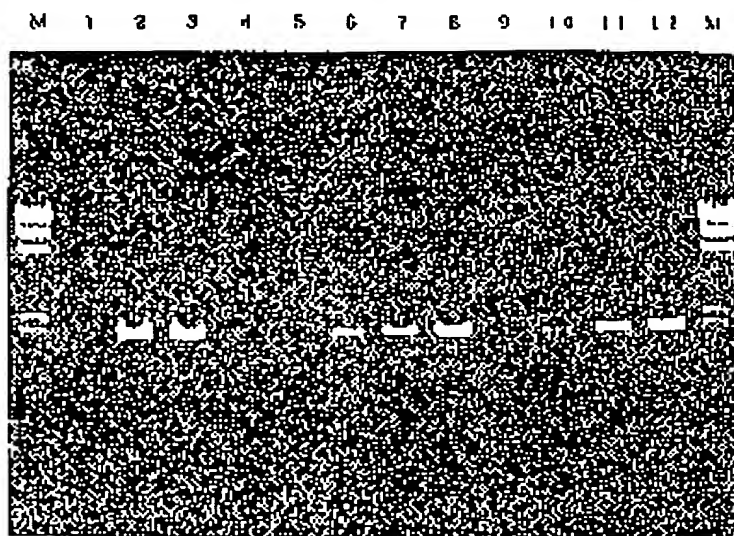


2 b

Deep Vent Polymerase		Taq Polymerase	
1:	Reaction Time: 1.5 min	1:	Reaction Time: 1.0 min
2:	" : 2.0 min	2:	" : 1.5 min
3:	" : 2.5 min	3:	" : 2.0 min
		4:	" : 2.5 min

Fig. 3

Comparison of the PCR due to the Difference in the Reaction Time
of Various Thermostable DNA Polymerases



- | | | |
|-----|--|-----------------------|
| 1: | KOD Polymerase (Derived from Hyperthermophilic archaeon strain KOD1) | Reaction Time: 1 sec. |
| 2: | KOD Polymerase (Derived from Hyperthermophilic archaeon strain KOD1) | " " : 5 sec. |
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| 4: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | Reaction Time: 1 sec. |
| 5: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 5 sec. |
| 6: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 10 sec. |
| 7: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 30 sec. |
| 8: | Taq Polymerase (Derived from <i>Thermus aquaticus</i>) | " " : 60 sec. |
| 9: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 30 sec. |
| 10: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 60 sec. |
| 11: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 90 sec. |
| 12: | Pfu Polymerase (Derived from <i>Pyrococcus furiosus</i>) | " " : 120 sec. |

Fig. 4

Construction of Expression Recombinant Vector (pET-pol)

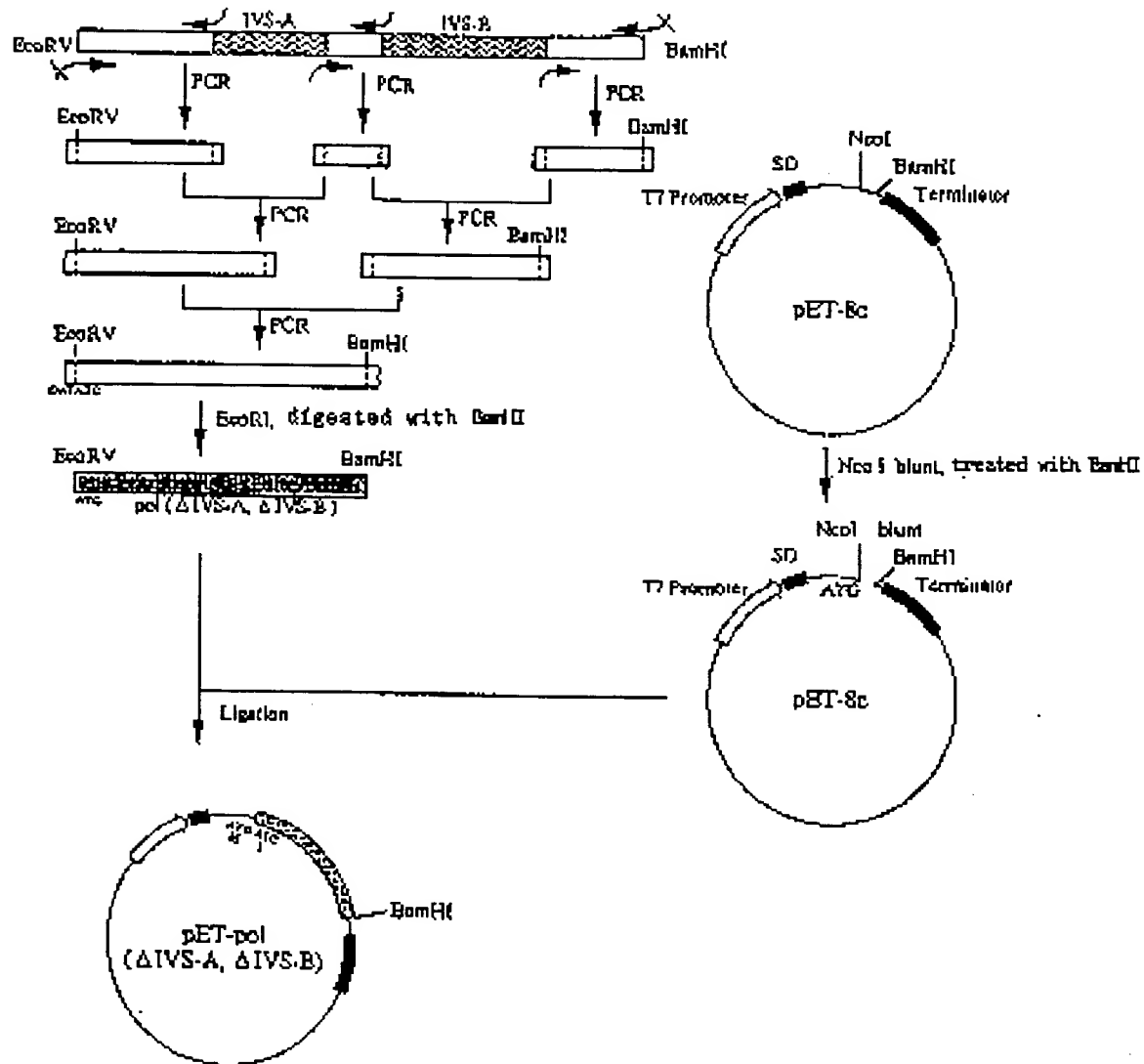
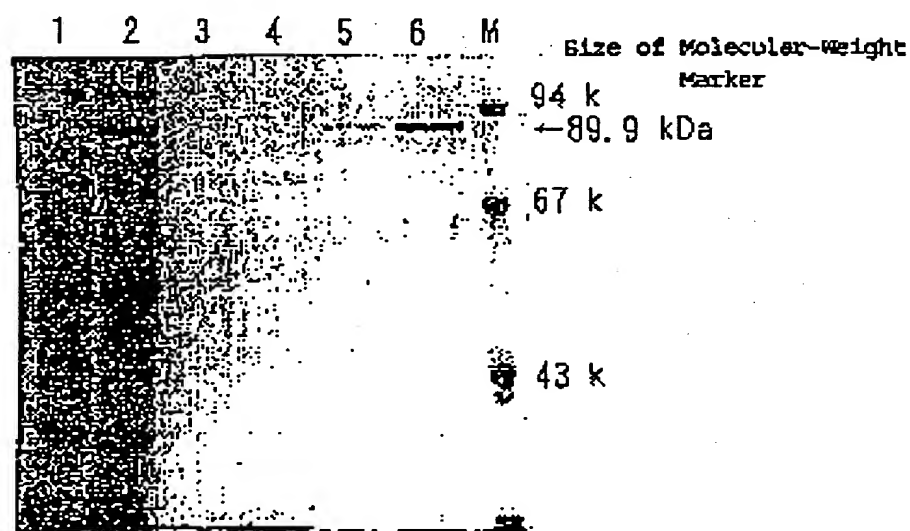
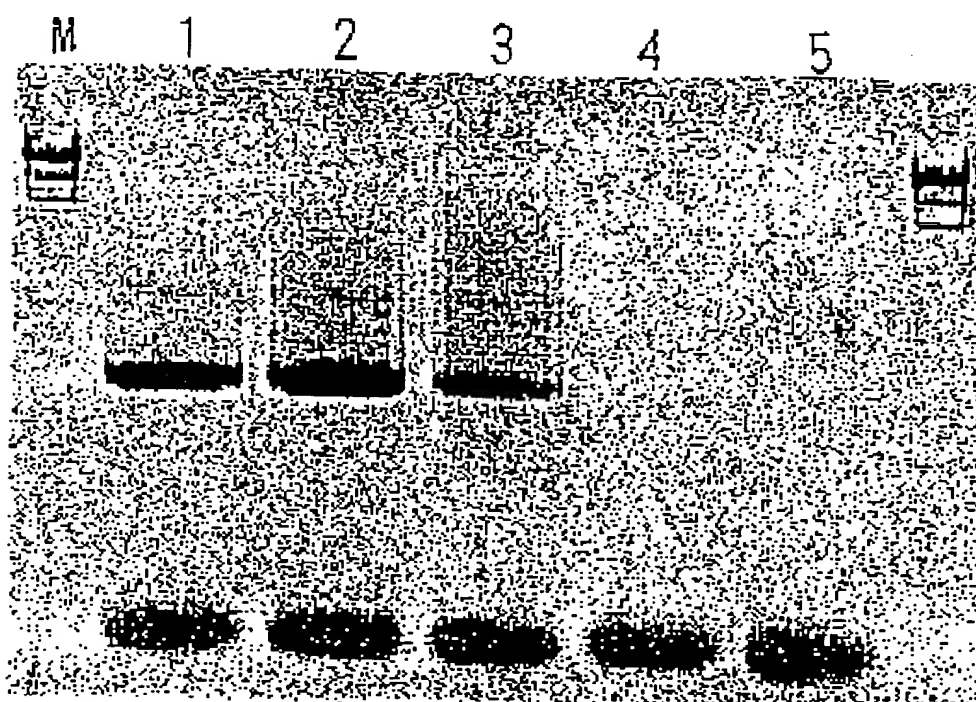


Fig. 5



- 1: pET-8c Precipitate
- 2: pET-pol (Δ IVS-A, Δ IVS-B) Precipitate
- 3: pET-8c Supernatant Liquid
- 4: pET-8c Supernatant Liquid x 5
- 5: pET-pol (Δ IVS-A, Δ IVS-B) Supernatant Liquid
- 6: pET-pol (Δ IVS-A, Δ IVS-B) Supernatant Liquid x 5

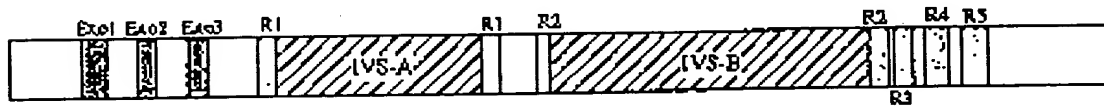
Fig. 6



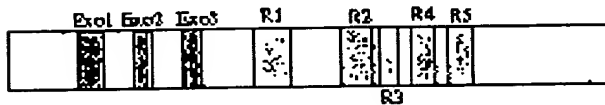
- 1: Vent Polymerase (Derived from *Thermococcus litoralis*)
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- 4: pET-8c Supernatant Liquid
- 5: pET-8c Supernatant Liquid x 5

Fig. 7

DNA Polymerase Gene of Hyperthermophilic Archaeon Strain KOD1

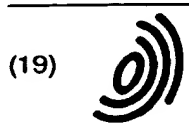


DNA Polymerase Gene of *Pyrococcus furiosus* (Pfu DNA Polymerase)



DNA Polymerase Gene of *Thermococcus litoralis* (Vent DNA Polymerase)





(19)

Europäisches Patentamt

European Patent Office

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(71) Applicant: Toyo Boseki Kabushiki Kaisha
Osaka-shi Osaka 530 (JP)

(72) Inventors:

- Kitabayashi, Masao,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
- Arakawa, Taku,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
- Inoue, Hiroaki,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)

- Kawakami, Bunsei,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
- Kawamura, Yoshihisa,
c/o Toyo Boseki K.K.
Tsuruga-shi, Fukui-ken (JP)
- Imanaka, Takayuki
Suita-shi, Osaka-fu (JP)
- Takagi, Masahiro
Suita-shi, Osaka-fu (JP)
- Morikawa, Masaaki
Minoo-shi, Osaka-fu (JP)

(74) Representative: VOSSIUS & PARTNER
Postfach 86 07 67
81634 München (DE)

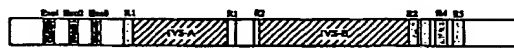
(54) A method of amplifying nucleic acid and a reagent therefor

(57) An object of this invention is to provide an enzyme which amplifies nucleic acid with a high fidelity within a short reaction time and also to provide a method of amplification.

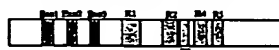
This invention relates to a thermostable DNA polymerase having at least 30 bases/second of DNA extension rate and a 3'-5' exonuclease activity derived from an hyperthermophilic archaeon strain KOD1, to a method of amplifying and also of detecting the nucleic acid using said enzyme and to a reagent kit used for those methods.

Fig. 7

DNA Polymerase Gene of Hyperthermophilic Archaeon Strain KOD1



DNA Polymerase Gene of Pyrococcus furiosus (Pfu DNA Polymerase)



DNA Polymerase Gene of Thermopoccus litoralis (Vent DNA Polymerase)



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European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 10 8613

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	DATABASE EMBL DNA-Sequence Data Library, Heidelberg, BRD creation AC D29671, 23 April 1994 XP002017309	1-5,10, 11	C12N15/54 C12N9/12 C12N1/21 C12N15/70 C12Q1/68 //(C12N1/21, C12R1:19)
Y	* abstract * & UNPUBLISHED, KAKIHARA H.: "Cloning and analysis of the DNA polymerase gene from a new hyperthermophilic archaeon, Pyrococcus sp. strain KOD1"	6-9, 12-20	
X	EP-A-0 624 641 (HOFFMANN-LA ROCHE) 17 November 1994	1,6,10, 15-20	
Y	* the whole document *	6-9, 12-20	
X	EP-A-0 547 920 (NEW ENGLAND BIOLABS, INC.) 23 June 1993	5	
A	* the whole document *	1-20	
P,X	FASEB JOURNAL, vol. 10, no. 6, 30 April 1996, page 1423 XP002017308 KITABAYASHI M.: "KOD DNA-Polymerase - Application for fast and accurate PCR" * the whole document *	1-20	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C12N C12Q
Place of search		Date of completion of the search	Examiner
THE HAGUE		30 October 1996	Kania, T
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure F : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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